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13. ABSTRACT ***(Maximum 200 Words)***

This is the annual report for the third year of DAMD-17-91-8509. The third year of the proposal has gone quite well. Aims 1 and 2 are essentialy complete. Moajor findings include 1) Confirmation that GJIC contributes to bone cell PGE2 but not $[Ca^{2+}]_i$ response to fluid flow. This was demonstrated in two different osteoblastic cell lines using several different techniques to inhibit GJIC. 2) Confirmation that ROS cells do not display an increase in $[Ca^{2+}]_i$ in response to fluid flow. 3)Discovery that the lack of a Ca^{2+} response to fluid flow in ROS may be due to lack of purinergic P2Y2 receptors. 4) Discovery that an ATP interaction with purinergic receptors contributes to fluid flow responsiveness in both ROS and MC3T3-E1 cells. 5) Discovery that there is a refractory period for fluid flow induced calcium mobilization in osteoblastic cells. The last year of the project will focus on completion of aim 3.

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Introduction

Bone cells are normally found in voids in the mineralized matrix known as lacunae. Small tubes in the matrix, known as canaliculi, interconnect the lacunae and are occupied by cellular processes. Gap junctions form where the processes of neighboring cells come into contact allowing for cell-cell communication via signaling substances. The extracellular space between the cell membrane and the mineralized matrix is filled with fluid which communicates with the bone's vascular supply. As the bone matrix is cyclically loaded due to physical activity, fluid flows in the lacunar-canalicular network from regions of high matrix strain to regions of low matrix strain and back again. Physiologic levels of this oscillating fluid flow have been shown to be a potent stimulator of bone cells *in vitro*. Additionally, we have shown that flow induced signals are transmitted from cell to cell such that cells that are not responding to flow directly can indirectly respond to flow-induced signals transmitted via gap junctions from adjacent cells. The consequence of this is that an ensemble of cells coupled via gap junctions is more sensitive to the effects of fluid flow than an equivalent collection of uncoupled cells. Our central hypothesis is that *there is an age-related decrease in the cellular responsiveness to fluid flow which is compounded by a decrease in cell-cell communication.*

In the first year of funding we made the following key findings:

- Discovered that neither ROS cells nor the RCx16 and ROS/Cx45 transfectants of ROS cells exhibit increase $[Ca^{2+}]_i$ in response to oscillating fluid flow.
- Discovered that ROS cells as well as the RCx16 and ROS/Cx45 transfectants of ROS cells release PGE2 in response to oscillating fluid flow.
- Accumulated early evidence that cell-cell communication via gap junctions plays an important role in regulating bone cell anabolism.

In the second year of funding we made the following key findings:

- Discovered that RCx16 and ROS/Cx45 gap junction deficient cells are less responsive to oscillatory fluid flow in terms of PGE2 release than ROS cells.
- Discovered that MC3T3-E1 cells exhibit an increase $[Ca^{2+}]_i$ in response to oscillating fluid flow, regardless of gap junctional intercellular communication (GJIC).
- Discovered that MC3T3-E1 cells release PGE2 in response to oscillating fluid flow via a mechanism that involves GJIC.
- Confirmed that the PGE2 response to oscillating fluid flow does not require a $[Ca^{2+}]_i$ response.
- Demonstrated that cell-cell communication via gap junctions plays an important role in regulating bone cell anabolism.
- Discovered that confluent and subconfluent cultures of rat osteoblastic cells have functional GJIC that is not age dependent.
- Discovered that the percentage of cells displaying fluid flow induced intracellular calcium signaling in ensembles of rat osteoblastic cells was significantly greater in cells from mature rats (12 mos.) than in cells from old rats (24 mos.) for an array of functional loading regimes.

Body

Aim 1: (Year 1) Demonstrate that the responsiveness of a cell ensemble is related to the degree of cell-cell communication. Initially we proposed to examine this aim using three immortalized cell lines ROS (rat osteosarcoma), RCx16, and ROS/Cx45. Both RCx16 and ROS/Cx45 cells are ROS cells that have been transfected (but following different strategies) to limit GJIC. Previously we have shown that RCx16 and ROS/Cx45 cells are less coupled than ROS cells. We found that none of the ROS lines

responded to either steady or oscillatory fluid flow with an increase in $[Ca^{2+}]_i$ This was true at several different flow rates and with perfusate media containing 2%, 5%, or 10% fetal bovine serum. However, ROS cells did respond to oscillatory fluid flow with an increase in PGE2 accumulation whereas the poorly coupled RCx16 and ROS/Cx45 did not. This suggest that the PGE2 response to fluid flow was dependant on GJIC. However, because the ROS cells do not demonstrate an increase in Ca^{2+} in response to fluid flow we were unable to determine the role of GJIC in this process. To address this issue we completed experiments utilizing MC3T3-E1 cells expressing a dominant/negative Cx43. Wild type MC3T3-E1 cells responded to fluid flow with an increase in Ca^{2+} and are thus an appropriate model to examine the role of GJIC in the Ca^{2+} response to fluid flow.

The detailed results of our MC3T3-E1 experiments are contained in the published manuscript Saunders et al AJP Cell 281:C1917-1925, 2001 (appendix 1). In summary we demonstrated that GJIC assessed by dye diffusion is diminished in the genetically manipulated cell line (DN-8) after 96 hours in culture, but not prior at 48 and 24 hours in culture. The control and DN-8 cells exhibited equivalent $[Ca^{2+}]_i$ responsiveness to flow, suggesting that $[Ca^{2+}]_i$ responsiveness to flow does not involve GJIC. In contrast, we found dramatically diminished release of PGE2 in response to flow in the coupling deficient DN-8 cells (96 hours) suggesting that the PGE2 response to flow does depend on GJIC.

These findings raised the possibility that the PGE2 response to flow does not involve a flow-induced increase in $[Ca^{2+}]_i$. To verify this interpretation we conducted an additional series of experiments using a blocker of intracellular cytosolic calcium mobilization. The results of these experiments demonstrate that PGE2 release in response to fluid flow can occur without a $[Ca^{2+}]_i$ increase. This conclusion is also consistent with our findings in the previous experiments utilizing ROS cells where we observed a PGE2 response to flow in a cell line that did not exhibit a $[Ca^{2+}]_i$ response to flow. This conclusion is highly significant since it suggests that the fluid flow response (and specifically that to oscillatory fluid flow) is unique in that PGE2 release can occur without the $[Ca^{2+}]_i$ response, which has not been previously observed for other physical and hormonal stimuli.

We have also initiated experiments to examine the surprising finding that ROS cells do not respond to fluid flow with an increase in Ca^{2+}. Previous studies suggest that ROS cells do not express the purinergic receptor P2Y2. This receptor has been implicated in ATP regulation of Ca^{2+} in bone cells. Furthermore, ATP has been implicated in the mechanism by which fluid flow increases $[Ca^{2+}]_i$ in endothelial cells. Therefor we examined the hypothesis that the lack of P2Y2 in ROS cells at least partially contributes to their inability to increase $[Ca^{2+}]_i$ in response to fluid flow. To examine this hypothesis we obtained ROS cells transfected with P2Y2 cDNA (ROS/P2U). We found that only 25.8% of wild type ROS 17/2.8 cells lacking P2Y2 receptors responded with an increase in $[Ca^{2+}]_i$ whereas 69.0% of ROS/P2U cells responded. This suggest that the P2Y2 receptor, and therefore ATP, contributes to fluid flow induced increases in $[Ca^{2+}]_i$ in ROS cells. To determine whether this is the case in other osteoblastic cells we exposed MC3T3-E1 cells to oscillating fluid flow in the presence of P2Y2 antisense oligonucleotides. We found that P2Y2 antisense oligonucleotide treatment decreased the percentage of cells responding to flow with an increase in $[Ca^{2+}]_i$, relative to scrambled P2Y2 oligonucleotide treatment (29.0% vs. 65.3% respectively). Taken together these results suggest that whereas GJIC does not appear to be critical for fluid flow induced increases in $[Ca^{2+}]_i$ a functional P2Y2 purinergic receptor is. This suggests that nucleotides such as ATP contribute to fluid flow responsiveness in bone cells. These results will be presented at the Orthopaedic Research Society Meeting in February 2002 (appendix 2).

Aim 2: (Years 2 and 3) Quantify the responsiveness of bone cells as a function of age and cell-cell communication. Bone cells will be cultured from young, mature, and old rats and exposed to the fluid flow protocol of Aim 1. We have demonstrated that confluent cultures of rat osteoblastic cells (ROB) from young, mature, and old animals display highly functional GJIC. We found comparable functional communication in subconfluent ROB, from all three age groups, that were seeded on quartz microscope slides for calcium analysis.

Fluid flow induced shear stress was used as a mechanical stimulus to study intracellular calcium signaling in ROB that were isolated from young, mature, and old animals. Fura-2 was used to measure $[Ca^{2+}]_i$ in cells that were exposed to three minutes of oscillating fluid flow that produced shear stresses of 1 or 2 Pascals (Pa) at frequencies of 0.2, 1, or 2 Hz. Fluid flow caused an immediate and transient increase in $[Ca^{2+}]_i$. A significantly higher percentage of mature ROB displayed calcium transients than old ROB. Cells were more responsive to 0.2 Hz than to 1 or 2 Hz, and to 2 Pa than 1 Pa. These data suggest that intracellular calcium signaling is an important mechanotransduction response in rat osteoblastic cells and that there are age-related as well as frequency and shear stress amplitude dependent responses to oscillatory fluid flow. This work has recently been published in Donahue et al AJP Cell 281:C1635-C1641, 2001 (appendix 3).

With support from this grant we have been able to examine other aspects of fluid flow affects on osteoblasts not directly related to our original hypothesis but with important implications regarding bone mechanotransduction. Previous studies suggest that bone cells exposed to mechanical signals display a refractory period, during which they are insensitive to additional mechanical stimuli. This may partially explain why intermittent loading is more anabolic than continuos loading. To examine the hypothesis that bone cells display a refractory period we exposed rat osteoblasts to sequential bouts of fluid flow separated by various time periods. We found that while a small percentage of cells could respond to a second bout of fluid flow after only a 5 second rest period, significantly ($p < 0.04$) fewer cells displayed $[Ca^{2+}]_i$ oscillations when the rest period was less than 600 seconds. Additionally, the magnitude of the second $[Ca^{2+}]_i$ oscillation was significantly ($p < 0.01$) lower than the magnitude of the first $[Ca^{2+}]_i$ oscillation for rest periods less than 900 seconds. As many as four fluid flow induced $[Ca^{2+}]_i$ oscillations could be invoked when rest periods of 2700 seconds were given between each bout. However, during one hour of continuous oscillating fluid flow, no subsequent $[Ca^{2+}]_i$ oscillations were observed after the initial immediate response. These findings suggest that a rest period is required for multiple fluid flow induced $[Ca^{2+}]_i$ responses in osteoblastic cells, but the refractory period may be as short as 5 seconds for some individual cells. However, a 900 second rest period was required to recover both the percentage of cells responding and the magnitude of the response. *In vivo*, rest periods enhance mechanically induced bone formation. Therefore, it is reasonable to hypothesize that $[Ca^{2+}]_i$ oscillations play a role in *in vivo* bone adaptation. These results have will be presented at the Orthopaedic Research Society meeting in February 2002 (appendix 4) and have been submitted for publication in the Journal of Biomechanics (appendix 5).

Aim 3: (Years 3 and 4) Examine the effect of forskolin on fluid flow responsiveness in osteoblasts as a function of age. The last year of the project will focus on this aim.

Key Research Accomplishments

- Confirmed that GJIC contributes to bone cell PGE2 but not Ca^{2+}_i response to fluid flow. This was demonstrated in two different osteoblastic cell lines using several different techniques to inhibit GJIC.
- Confirmed that ROS cells do not display an increase in $[Ca^{2+}]_i$ in response to fluid flow.
- Discovered that the lack of a Ca^{2+} response to fluid flow in ROS may be due to lack of purinergic P2Y2 receptors.
- Discovered that ATP interaction with purinergic receptors contributes to fluid flow responsiveness in both ROS and MC3T3-E1 cells.
- Discovered that there is a refractory period for fluid flow induced calcium mobilization in osteoblastic cells.

Reportable Outcomes

The following manuscripts and abstracts were supported either partially or fully by the project:

1. Yellowley, C.E., Jacobs, C.R. and Donahue, H.J. (1999) Mechanisms contributing to fluid-flow-induced Ca^{2+} mobilization in articular chondrocytes. Journal of Cellular Physiology. 180(3):402-8.

2. Yellowley, C.E., Li, Z., Zhou, Z., Jacobs, C.R. and Donahue, H. J. (2000) Functional gap junctions between osteocytic and osteoblastic cells. J. Bone Min Res. 15(2)209-217.

3. You, J., Yellowley, C.E., Donahue, H.J., Zhang, Y., Chen Q., and Jacobs, C.R. (2000) Substrate deformation levels associated with routine physical activity are less stimulatory to bone cells relative to loading induced oscillatory fluid flow. J. Biomech. Eng. 122:387-393.

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5. Kurokouchi, K., Jacobs, C.R., Donahue, H.J. (2001) Oscillating fluid flow inhibits tumor necrosis factor-α-induced nuclear factor-κB activation via IκB kinase pathway in osteoblast-like UMR106 cells. J. Biol. Chem. 276(16)13499-13504.

6. You, J., Reilly, G.C., Zhen, X., Yellowley, C.E., Chen, Q., Donahue, H.J., Jacobs, C.R. (2001) Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. J. Biol. Chem. 276(16)13365-13371.

7. Saunders, M.M., You, J., Trosko, J., Tamasaki, H., Donahue, H.J., Jacobs, C.R. (2001) Gap junctions and fluid flow response in MC3T3-E1 cells. Amer. J. Physiol. Cell 281:C1917-C1925.

8. Donahue, S.W., Jacobs, C.R., Donahue, H.J. (2001) Flow induced calcium oscillations in rat osteoblasts are age loading frequency and shear stress dependent. Amer. J. Physiol. Cell Physiol. 281: C1635-C1641.

9. Buhl, K.M, Jacobs, C.R., Turner, R.T., Evans, G.L., Farrell, P.A., Donahue, H.J. (2002) Parallel changes in extracellular matrix protein gene expression, bone formation and biomechanical properties in aging rat bone. Bone (in review).

10. Alford, A.I., Jacobs, C.R., Donahue, H.J. (2002) Oscillating fluid flow regulates gap junction communication in osteocytic MLO-Y4 cells by an ERK1/2 MAP kinase dependent mechanism. J. Bone Min. Res. (in review).

11. Yellowley, C.E., Hancox, J.C., Donahue, H. J. (2002) Effects of cell swelling on intracellular calcium and membrane currents in bovine articular chondrocytes J. Bone Min. Res. (in review).

12. Donahue, S.W., Donahue, H.J. and Jacobs, C.R. (2002) Temporal aspects of fluid flow induced intracellular calcium oscillations in osteoblastic cells. J. Biomechanics (in review).

Abstracts

1. Saunders, M.M., You, J., Yellowley, C.E., Jacobs, C.R. and Donahue, H.J. (2000) Differential effect of oscillating flow on cytosolic calcium and prostaglandin in osteoblastic ROS 17/2.8 cells. 46th Annual Meeting of the Orthopaedic Research Society.

2. You, J, Yellowley, C.E., Donahue, H.J. and Jacobs, C.R. (2000) Fluid flow induced calcium mobilization is frequency dependent. 46th Annual Meeting of the Orthopaedic Research Society.

3. You, J., Zhen, X., Yellowley, C.E., Chen, Q., Donahue, H.J. and Jacobs, C.R. (2000) Mechanotransduction in bone cells via oscillating flow. 46th Annual Meeting of the Orthopaedic Research Society.

4. Kurokouchi, K. and Donahue, H.J. (2000) Oscillating fluid flow decreases TNF-α induced NF-κB activation in osteoblastic cells. 46th Annual Meeting of the Orthopaedic Research Society.

5. Saunders, M.M., Seraj, M.J.,Yellowley, C.E., Hoke, A., Welch, D.R., and Donahue, H.J. (2000) Gap junctional intercellular communication is restored in metastasis suppressed breast carcinoma cells. Experimental Biology 2000.

6. Yellowley, C.E., Jacobs, C.R., Donahue, H.J. (2000) Cell swelling induced activation of membrane currents and intracellular calcium in bovine articular chondrocytes. Society for Physical Regulation in Biology and Medicine.

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10. Saunders, M.M., You, J., Yellowley, C.E., Donahue, H.J. and Jacobs, C.R. (2000) Prostaglandin E_2 response to fluid flow is independent of intracellular calcium concentration in osteoblastic ROS 17/2.8 and RCx16 cells , Annals of Biomedical Engineering, Trans. BMES Annu. Meet. Abstract Supplement 1 (Trans. BMES Annu. Meet.), Vol. 28, S-87. 108.

11. Reilly, G.C., Yellowley, C.E., Donahue, H.J. and Jacobs, C.R. (2000) Investigation of the signalling pathways involved in the calcium response of bone cells to oscillating fluid flow. Journal of Bone and Mineral Research p S508.

12. You, J., Saunders, M..M., Yellowley, C.E., Donahue, H.J. and Jacobs, C.R. (2001) Oscillatory flow stimulates PGE2 release via protein kinase A in MC3T3-E1 osteoblasts involving cyclooxygenase-2. 47th Annual Meeting, Transactions of the Orthopaedic Research Society, Vol.26: p326.

13. Saunders, M..M., You, J., Trosko, J., Yamasaki ,H., Donahue, H.J. and Jacobs, C.R. (2001) Oscillatory fluid flow-induced prostaglandin E2 production is dependent upon gap junctional intercellular communication in osteoblastic MC3T3-E1 cells. 47th Annual Meeting, Transaction of the Orthopaedic Research Society, Vol.26: p565, 2001.

14. Saunders, M.M., Kunze, E., Li, Z., Mastro, A., and Donahue H.J. (2001) Quantification and characterization of gap junctions and gap junctional intercellular communication in an in vitro breast carcinoma model metastatic to bone. 47th Annual Meeting, Transaction of the Orthopaedic Research Society, Vol.26.

15. You, J., Saunders, M.M., Yellowley, C.E., Donahue, H.J., and Jacobs, C.R. (2001) Oscillatory flow-induced prostaglandin E2 release involves protein kinase a and cyclooxygenase-2 in MC3T3-E1 osteoblasts, Proceeding of the 2001 ASME Summer Bioengineering Conference, BED-Vol.50: p163-164.

16. Jacobs, C.R., You, J., Reilly, G.C., Saunders, M.M., Kurokouchi, K., Yellowley, C.E.and Donahue, H.J. (2001) An overview of oscillatory fluid flow as a potent loading-induced physical signal in bone, Proceeding of the 2001 ASME Summer Bioengineering Conference, BED-Vol.50: p339-340.

17. You, J., Saunders, M.M., Yellowley, C.E., Donahue, H.J. and Jacobs, C.R. (2001) Oscillatory flow-induced PGE2 release involves protein kinase A and cyclooxygenase2 in MC3T3-E1 osteoblasts. SPRBM 20th Annual Meeting, Charleston, SC.

18. Alford, A.I., Jacobs, C.R. and Donahue, H.J. (2001) Oscillating fluid flow modulates gap junction communication in the osteocyte-like cell line MLOy-4. Annals of Biomedical Engineering 29(S1)S19.

19. You, J., Jacobs, C.R., Donahue, H.J. (2001) Oscillatory fluid flow induces calcium mobilization by ATP/UTP via P2Y receptors in osteoblasts. Annals of Biomedical Engineering 29(S1)S25.

20. Donahue, S., Donahue, H.J. and Jacobs, C.R. (2001) Refractory period for fluid flow induced intracellular calcium oscillations in osteoblastic cells. Annals of Biomedical Engineering 29(S1)S28.

The following applications for funding were awarded by the NIH based on work supported this award:

"Mechanotransduction in bone via oscillating fluid flow", PI: C Jacobs, 1 R01 AR45989-01A1.

"Gap junctions and bone cell response to physical signals", PI: H Donahue, 2 R01 AG13087-06.

The following application is pending:

"The effects of extracellular nucleotides on osteblastic cells", PI: J. You

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Henry J. Donahue, Ph.D.
Christopher R. Jacobs, Ph.D.
Seth Donahue, Ph.D.
Marnie M. Saunders, Ph.D.
Jun You, Ph.D.

Conclusions

In summary, in the third year of the project we have completed aim one and two. For aim one we have demonstrated our hypothesis to be true, namely that GJIC modulates the PGE2 response of bone cells to oscillating fluid flow. Interestingly, we found that PGE_2 release in response to oscillating flow does not appear to involve intracellular calcium. This has important implications because it indicates that other second messengers, such as the cyclic AMP pathway, may be important in transducing the fluid flow signal into the PGE_2 response. Additionally, the differential effect of oscillating fluid flow on $[Ca^{2+}]_i$ versus PGE_2 may prove to be a powerful tool in further investigations of the bone cell mechanotransduction pathway. We have also established that while the $[Ca^{2+}]_i$ response to fluid flow does decrease as a function of age, GJIC does not. Our success with the first two aims have led to other interesting findings. For instance, we have demonstrated that a lack of the purinergic receptor P2Y2 is at least partially responsible for the inability of fluid flow to mobilize Ca^{2+} in ROS cells. This has led to our investigation of the role of ATP in mechanotransduction in bone. This is proving to be a very exciting area of research. Research supported by this funding has resulted in 8 manuscripts published, 3 of which directly relate to the specific aims and 5 of which are indirectly related to the aims. Additionally 3 manuscripts are in review. Finally, the results have been incorporated into two applications to the NIH which are now in their second year and one application for an NIH National Research Service Award which is pending.

Am J Physiol Cell Physiol
281: C1917–C1925, 2001.

Gap junctions and fluid flow response in MC3T3-E1 cells

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Saunders, M. M., J. You, J. E. Trosko, H. Yamasaki, Z. Li, H. J. Donahue, and C. R. Jacobs. Gap junctions and fluid flow response in MC3T3-E1 cells. *Am J Physiol Cell Physiol* 281: C1917–C1925, 2001.—In the current study, we examined the role of gap junctions in oscillatory fluid flow-induced changes in intracellular Ca^{2+} concentration and prostaglandin release in osteoblastic cells. This work was completed in MC3T3-E1 cells with intact gap junctional communication as well as in MC3T3-E1 cells rendered communication deficient through expression of a dominant-negative connexin. Our results demonstrate that MC3T3-E1 cells with intact gap junctions respond to oscillatory fluid flow with significant increases in prostaglandin E_2 (PGE_2) release, whereas cells with diminished gap junctional communication do not. Furthermore, we found that cytosolic Ca^{2+} (Ca_i^{2+}) response was unaltered by the disruption in gap junctional communication and was not significantly different among the cell lines. Thus our results suggest that gap junctions contribute to the PGE_2 but not to the Ca_i^{2+} response to oscillatory fluid flow. These findings implicate gap junctional intercellular communication (GJIC) in bone cell ensemble responsiveness to oscillatory fluid flow and suggest that gap junctions and GJIC play a pivotal role in mechanotransduction mechanisms in bone.

prostaglandin E_2; calcium; mechanotransduction; gap junctional intercellular communication

IT IS WIDELY ACCEPTED that bone adapts to its physical loading milieu by optimizing mass and mechanical performance in a process known as bone remodeling. In a nonpathological scenario, this process results in normal bone turnover whereby new bone formation is balanced by removal of existing bone. In a pathological scenario, this process results in an imbalance whereby net bone formation (osteopetrosis) or net bone loss (osteopenia) ensues. Although the effects of remodeling have been histologically observed, the exact cellular pathways by which it occurs are incompletely understood. To this end, researchers have recently begun to investigate mechanotransduction mechanisms (15, 17, 32) in an attempt to better uncover the elusive signal transduction pathways by which physical stimuli can affect cellular responses in bone. These studies have found that bone cells can respond to a wide variety of endogenously occurring signals including mechanical stretch (41), streaming potentials, chemotransport, electrical effects (2, 6, 26, 33), and fluid flow (8, 17, 28, 41). In the latter area of research, it has been hypothesized that the fluid flow through the lacunar-canalicular network is pivotal to bone cell responsiveness. Although several hypotheses have been proposed, many believe that the osteocytes in the canalicular spaces sense the fluid flow (1) and in turn signal the osteoblasts to form bone.

Although many accept the theory that osteoblast responsiveness to biophysical effects is linked to the osteocyte (1, 23), few have proposed a mechanism by which this may occur. We propose that bone cell responsiveness to fluid flow is aided by gap junctions that physically connect osteoblasts and osteocytes (39) as well as osteoblasts to other osteoblasts. In this scenario, we hypothesize that gap junctions not only enable osteocytes to transfer signals to osteoblasts but that the responsiveness of osteoblastic networks to the signal is amplified via gap junctions. By coupling the osteoblasts together, gap junctions enable the cells to respond in concert, resulting in a more robust response attained than if an equal number of individual cell responses was achieved. This is the focus of our current work.

Gap junctions are transmembrane protein channels that enable neighboring cells to physically link, thereby facilitating the rapid diffusion of small molecules and ions on the order of 1 kDa in a process known as gap junctional intercellular communication (GJIC). Gap junctions may be homospecific, uniting cells of the same type, or may be heterospecific, uniting cells of unlike type. With the exception of blood cells and muscle fibers, gap junctions have been found in most cells (30), with at least 13 mammalian connexins identified to date and named with respect to molecular weight.

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There is growing evidence to support a role for gap junctions in the cellular (and cell ensemble) response to physical stimuli. In bone, gap junctions have been linked to such functions as hormonal responsiveness (34), gene expression (25), and differentiation (7). Intercellularly, gap junctions have been linked to second messenger responses induced by physical stimuli such as Ca^{2+} release following membrane deformation (21). Interestingly, many fluid flow studies have shown that osteoblastic cells respond to flow in vitro with an increase in such second messengers as Ca^{2+} (13, 15), cAMP (28), and NO release (20), which have been shown to be physical regulators of gap junction channel opening.

GJIC has been linked to both normal and abnormal cell function. In normal cell function, GJIC has been linked to such processes as proliferation and differentiation, although the findings at times have been inconsistent. For instance, while GJIC is found to maintain cell differentiation status in cultured hepatocytes (40), it is decreased in differentiating keratinocytes compared with proliferating ones (12). Furthermore, we have recently demonstrated that gap junctional function and expression parallel osteoblastic differentiation, contributing to alkaline phosphatase expression (7). Thus gap junction studies are widely dependent on cell line, culture conditions, and experimental environment, and results must be interpreted within these contexts. In abnormal cell function, alterations in GJIC have been linked to disease (30, 36), suggesting that a status quo in gap junctional function is crucial to homeostasis.

In the current study, we set out to examine the role of GJIC in transducing a mechanical stimulus to bone cells. That is, we exposed osteoblastic cells to levels of oscillatory fluid flow that occur in vivo due to habitual loading (35), and we measured prostaglandin E_2 (PGE_2) release and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), markers selected for their proposed role in the regulation of bone turnover (4, 10, 16, 19, 27). To correlate these findings with the role of GJIC, we utilized osteoblastic MC3T3-E1 cells, MC3T3-E1 cells expressing a dominant-negative connexin43 (Cx43; DN-8), the predominant gap junction protein in bone, and a control transfectant (DN-VC). Comparisons of changes in PGE_2 release and $[Ca^{2+}]_i$ in the presence of oscillatory fluid flow in the three cell lines were then used to draw conclusions about the contribution of gap junctions and GJIC to bone remodeling.

MATERIALS AND METHODS

Cell culture. Three immortalized osteoblastic cell lines were utilized in this study: MC3T3-E1, DN-VC, and DN-8. The MC3T3-E1 is an immortalized mouse osteoblastic cell line; the DN-8 is a neomycin-sustained transfectant of MC3T3-E1 containing a mutant Cx43; and the DN-VC is a control for the transfection containing an empty plasmid. MC3T3-E1 cells were cultured in Eagles's minimal essential medium (MEM-α; GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% penicillin/streptomycin (GIBCO BRL). The DN-8 and DN-VC cells were cultured in MC3T3-E1 medium supplemented with neomycin (200 μg/ml). All cell lines were maintained in an incubator at 37°C and 5% CO_2, with flow experiments conducted in the appropriate media supplemented with 2% FBS.

The DN-8 line was developed from a dominant-negative strategy as previously described (24). Briefly, a mutant gap junction protein (Cx43Δ) of Cx43 was developed by the deletion of residues in the internal cytoplasmic loop of the connexin structure. The goal of this strategy was to introduce this mutant gene into both protein channels of each linking cell such that the mutant could oligomerize with only a wild-type species. Unlike previous dominant-negative strategies in which GJIC is obliterated and the resulting connexin oligomers are not transported to the membrane but remain in the cytoplasm, this novel mutation approach affects only permeability, leaving transport intact (24).

Cell preparation. Experiments were conducted on two differently sized microscope slides. For the quantification of oscillatory fluid flow-induced cytosolic Ca^{2+} (Ca_i^{2+}) mobilization, cells were plated on quartz slides (76 mm × 26 mm × 1.6 mm) for imaging. These slides accommodated the relatively few cells needed to conduct the experiments and were made of quartz to allow for ultraviolet visualization. Cells were plated at 1.0×10^5, 0.75×10^5, or 0.5×10^5 cells/slide and cultured for 24, 48, or 96 h, respectively, to achieve 85–90% confluence. For the quantification of oscillatory fluid flow-induced PGE_2 production, cells were plated on glass microscope slides (75 mm × 38 mm × 1 mm). These slides were larger so that larger volumes of cells could be evaluated. Cells were plated at 3.5×10^5, 2.75×10^5, or 2.0×10^5 cells/slide and cultured for 24, 48, or 96 h, respectively, to achieve 85–90% confluence. For quantification of GJIC, cells were plated as described for the PGE_2 experiments. In addition, cells for double labeling were cultured in round (35-mm-diameter) polystyrene petri dishes in the appropriate media for 24, 48, or 96 h.

GJIC assays. GJIC assays were completed using epifluorescent microscopy and a double-labeling technique, as previously described (39). In this technique, cells are loaded with the fluorescent dyes calcein AM (Molecular Probes, Eugene, OR) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes). The fluorescent dye calcein AM, once in the cell, is cleaved of its AM group and trapped within the cell. However, as a result of its small molecular size (<1 kDa), calcein is gap junction permeable and able to transfer to neighboring cells if functional (open) gap junctions are established. The fluorescent dye DiI is of a larger molecular size, intercalates within cell membranes, and does not transfer to neighboring cells via GJIC. The loaded cells are then dropped onto unloaded cells in a monolayer, and cell transfer is quantified. If functional gap junctions are established, the calcein will transfer to neighboring cells that will then fluoresce green.

Coupling assays were completed to establish the extent of disruption of GJIC in DN-8 cells at 24, 48, and 96 h in culture and were compared with GJIC in the MC3T3-E1 and DN-VC lines at the same time points. After quantification of GJIC, we assessed GJIC in the three cell lines at 96 h in culture simultaneously with PGE_2 and $[Ca^{2+}]_i$ experiments to minimize passage variables. On the day of the experiments, the preconfluent cells ("donor" cells) plated in the petri dishes were removed from the incubator and washed twice with room temperature phosphate-buffered saline (PBS) followed by aspiration. The donor cells were labeled with a BSA-enriched PBS-fluorescent dye mixture containing 20 μl of calcein AM, 7 μl of DiI, and 20 μl of pluronic acid (Molecular

Probes) and incubated for 30 min at 37°C. After being incubated, the dye mixture was aspirated, and the donor cells were washed twice in room temperature PBS. The donor cells were detached from the dishes by trypsinization, centrifuged at 200 *g* for 8 min, and resuspended in fresh growth medium. The double-labeled (calcein and DiI) donor cells were then dropped onto the glass slides containing confluent monolayers of unlabeled cells at a ratio of ~1:500 cells (labeled to unlabeled) and incubated for 90 min at 37°C. After the incubation period, the slides were removed from the dishes, washed twice with PBS, and covered by round (25-mm-diameter) glass coverslips. The slides were placed on a Nikon fluorescent microscope (Nikon EFD-3; Optical Apparatus, Ardmore, PA) and visualized using fluorescein ($\lambda_{excitation}$ = 465–495 nm; $\lambda_{emission}$ = 520 nm) and rhodamine ($\lambda_{excitation}$ = 541–551 nm; $\lambda_{emission}$ = 590 nm) filters to locate the calcein- and DiI-loaded cells, respectively. Coupling was quantified by counting the number of neighboring cells fluorescing green, while the DiI was used to distinguish the labeled cells from those in the monolayer. Thirty cells were randomly selected and counted for each slide. Coupling was considered extensive if individual cells transferred calcein to >15 cells and were not counted past this threshold number.

Parallel plate flow chambers and testing machine. For PGE_2 and Ca^{2+} experiments, bone cells were placed in a parallel plate flow chamber and subjected to oscillatory fluid flow. This system has been previously characterized, and we and others have employed it to expose endothelial cells (8), chondrocytes (37, 38), and bone cells (14, 15, 17, 41) to physiological levels of fluid flow. Briefly, the system imparts a laminar flow to the cells in a monolayer, exposing them to a shear stress governed by the equation (9)

$$\tau = 6\mu Q/bh^2$$

where τ is the shear stress, μ is the viscosity of the flow medium, Q is the flow rate, and b and h are the width and height of the chamber, respectively. To accommodate the quartz and glass microscope slides, as previously noted, two differently sized chambers were employed. In general, the components for both chambers were the same and are shown in the exploded view of Fig. 1. The chambers consisted of a polycarbonate manifold, a silastic gasket, and a glass slide. This slide containing the cells in a monolayer formed the bottom of the flow chamber when inverted on the manifold. For Ca^{2+} studies, an 18 ml/min flow rate resulted in a shear

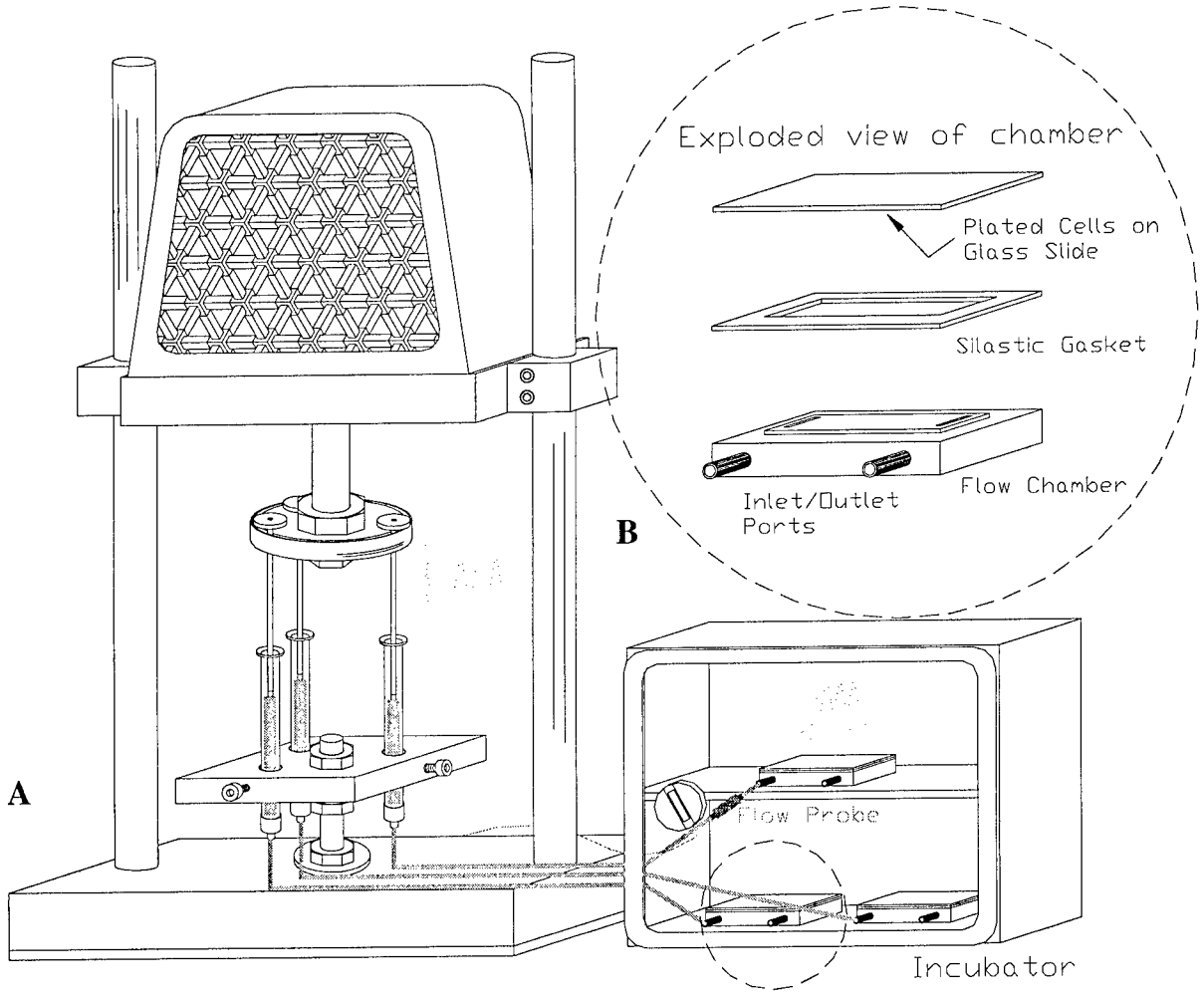

Fig. 1. Schematic of oscillatory fluid flow delivery system. *A*: oscillatory fluid flow was delivered via a sinusoidal waveform generated by a materials testing machine connected to the flow chamber using tubing and syringes. *B*: the flow chamber consisted of a parallel plate design. Cells in a monolayer on the glass slide were inverted on the flow chamber on a silastic gasket. During fluid flow, the assembly was held together with either a vacuum seal or encased in a polycarbonate case (not shown).

stress of 20 dyn/cm^2 and a rectangular flow volume of 38 mm × 10 mm × .28 mm; for PGE_2 studies, a 43 ml/min flow rate resulted in a shear stress of 20 dyn/cm^2 and a rectangular flow volume of 56 mm × 24 mm × 0.28 mm. In all flow experiments, flow rate was monitored with an ultrasonic flow probe (Transonic Systems, Ithaca, NY) connected to the chamber inlet. For Ca^{2+} imaging, the flow chamber assembly was held together with vacuum pressure; for PGE_2 quantification, the flow chamber assembly was placed in a polycarbonate case bolted together to form an air-tight seal. For the latter experiments, the polycarbonate case containing the chamber enabled the system to be placed in an incubator for long-term flow periods (1 h) such that temperature and CO_2 levels could be precisely regulated. For both short- (Ca^{2+}) and long-term (PGE_2) experiments, the chamber was connected to a pneumatic, closed-loop feedback materials testing machine (EnduraTec, Minnetonka, MN) via tubing and syringes with oscillatory fluid flow delivered in the form of a 1-Hz sine wave (Fig. 1).

PGE_2 quantification. PGE_2 accumulation in the supernatant was quantified with a commercially available, nonradioactive, competitive binding enzyme immunoassay system (BioTrak; Amersham Pharmaceuticals, Piscataway, NJ). After assay, the optical densities of the samples were read at 450 nm using a microplate reader (Dynex Technologies, Chantilly, VA). Manufacturer-supplied standards were also analyzed and used to construct a standard curve from which the sample concentrations were determined.

Oscillatory fluid flow-induced PGE_2 was quantified at the 48- and 96-h time points. On the day of the experiments, preconfluent slides of cells were washed, placed in the parallel plate flow chamber, encased in the polycarbonate case, placed in the incubator, and connected to the fluid flow delivery system. Cells were exposed to flow for 1 h, after which 10 ml of media from the inlet and outlet ports of the chamber and adjacent tubing were collected for PGE_2 analysis. These media are referred to throughout as media collected immediately postflow. In addition, the plates of cells were incubated in 10 ml of fresh medium for 1 h postflow, and these media were also collected for PGE_2 analysis. These media are referred to throughout as media collected 1 h postflow. Immediately after media collection, aliquots were frozen at −80°C. In addition, for some experiments, the ionophore 4-bromo-calcium (50 μM) was added to a plated slide from each cell line for 15 min at 37°C. The media from these collections were used as positive controls in the PGE_2 assays. On the day of assay, samples were thawed at 4°C and vortexed. Assays were completed at room temperature within 1 mo of collection, and degradation assays were completed to ensure that this time period did not adversely affect the results.

The three cell lines were also subjected to oscillatory flow in the presence of thapsigargin, a drug used in our study to empty and prevent refilling of intracellular Ca^{2+} stores, thus eliminating this source of Ca^{2+} contributing to changes in $[Ca^{2+}]_i$ (42). PGE_2 experiments in the presence of thapsigargin were completed at the 96-h time point following the exact protocol previously outlined with one exception: thapsigargin (50 nM) was added to each petri dish of plated cells (30 min before placing it in the flow chambers), the flow medium, and the 10 ml of fresh, 1-h postflow incubation medium.

Because total PGE_2 accumulation in the medium is dependent on cell number, prostaglandin accumulation was normalized to total cell protein for each slide. After the 1-h incubation and collection of the additional 10 ml of fresh medium, the cells were removed from each microscope slide by trypsinization, centrifuged at 200 *g* for 8 min, and resuspended in 0.5 ml of 0.05% Triton X-100 detergent. The suspended cells were placed in 1-ml centrifuge tubes and lysed using three cycles of rapid freezing (−80°C) and thawing. The lysate was frozen at −80°C until analysis with a commercially available assay kit (Bio-Rad, Hercules, CA). After assay, the optical densities of the samples were read at 405 nm using a microplate reader. Manufacturer-supplied standards were analyzed and used to construct the standard curve from which the sample concentrations were determined. Frozen cells and media were assayed at room temperature within 1 mo of collection.

Ca^{2+} imaging. Ca^{2+} imaging was completed with fluorescent microscopy and the dual-wavelenth ratiometric dye fura 2-AM (Molecular Probes). This indicator was selected for its ability to exhibit two distinct spectra and two distinct wavelengths based on the presence or absence of Ca^{2+} binding to the indicator (31). The indicator is loaded in the fura 2-AM form, which allows it to easily enter the cells. After loading, the AM groups are cleaved in an enzymatic process leaving the indicator trapped within the cell.

$[Ca^{2+}]_i$ was quantified in the three cell lines at the 96-h time point. On the day of the experiments, preconfluent slides of cells were loaded with 10 μM fura 2-AM in 1 ml of fresh media and incubated at 37°C for 45 min. After being incubated, the cells were washed in the appropriate flow medium (2% FBS), placed on the parallel plate flow chamber, transferred to a fluorescent microscope, and connected to the loading machine. To allow the cells to settle and ensure that the AM hydrolizing process was complete, the cells were allowed to equilibrate on the microscope stage for 30 min immediately before testing. Cells were subjected to 3 min of oscillatory flow preceded by a 3-min no-flow baseline. An image acquisition and analysis software package (Metafluor) was used to capture the images for $[Ca^{2+}]_i$ determination.

Data analyses. Ca^{2+} results were analyzed with a Rainflow counting technique (18). This technique, adapted from the field of mechanical fatigue, enables individual responses to be extracted from data containing multiple responses and has generally been employed to determine the contribution of a particular loading cycle to the overall lifetime of a structure. Rainflow applied to our research enabled individual cell responses to be isolated and separated from background noise with a threshold response defined as a change in $[Ca^{2+}]_i$ of ≥20 nM.

PGE_2 results were analyzed using a microplate reader and normalized to total protein with total PGE_2 accumulation in the medium given in picograms per micrograms. GJIC was quantified by counting cell fluorescence transfers, as previously described. All Ca^{2+}, PGE_2, and GJIC data obtained were expressed as means ± SE. To compare results among the cell lines, general linear model ANOVAs with Student-Newman-Keuls post hoc comparisons were completed using a commercially available software program (Instat; GraphPad Software, San Diego, CA) with an a priori significance level of 0.05.

RESULTS

Osteoblastic cell line GJIC as a function of time in culture. GJIC was qualitatively evaluated at 24-, 48-, and 96-h time points in the three cell lines with typical dye transfers shown (Fig. 2). In these double-exposed photographs, the green (calcein) fluorescence indicates the coupled cells in the monolayer, whereas the yellow (calcein and DiI) fluorescence indicates double-labeled donor cells. Quantitative results for the 24-, 48-, and

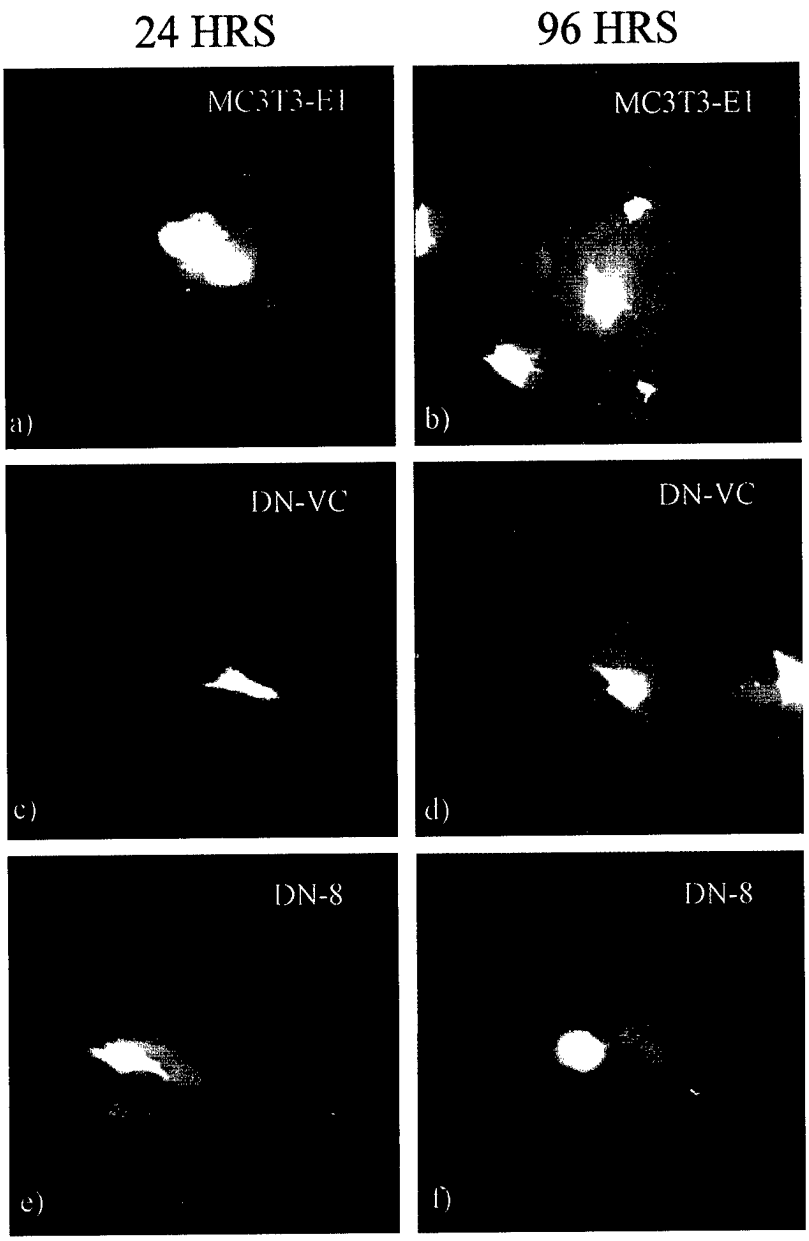

Fig. 2. Qualitative results of double-labeling assay at 24 and 96 h in the 3 cell lines examined. MC3T3-E1 (*A* and *B*), DN-VC (*C* and *D*), or DN-8 (*E* and *F*) cells were grown in the monolayer for 24, 48 (not shown), or 96 h and subjected to homospecific gap junctional intercellular communication analysis. Donor cells double labeled with the fluorescent dyes calcein and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were placed in contact with unloaded like cells in the monolayer. Cell transfer was visualized after 90 min. In the dual-exposure photographs, the cells fluorescing green (calcein) are the unlabeled cells in the monolayer demonstrating functional coupling; the cells fluorescing yellow (calcein and DiI) are the dual-labeled donor cells (original magnification, ×400).

96-h time points (Fig. 3) depict the number of donor cells coupled to individual acceptor cells in the monolayer. We found that the MC3T3-E1 and DN-VC cell line coupling was not dependent on time in culture up to 96 h. At 24 and 48 h, the three cell lines did not exhibit a significant difference in coupling compared with each other. However, at the 96-h time point, DN-8 cells exhibited a significant decrease in coupling compared with the MC3T3-E1 ($P < 0.001$) and DN-VC ($P < 0.001$) lines at 96 h, as well as compared with themselves at the 24- ($P < 0.001$) and 48-h ($P < 0.001$) time points.

PGE_2 accumulation in response to fluid flow. Because GJIC was decreased in DN-8 cells only after 96 h in culture, we first examined PGE_2 response to fluid flow at this time point (Fig. 4*A*). Media from MC3T3-E1 and DN-VC cells collected 1 h postflow accumulated significantly more PGE_2 than cells not exposed to flow ($P < 0.0005$ and $P < 0.0001$, respectively). However, media from poorly coupled DN-8 cells did not accumulate more PGE_2 than control cells. Similar results were obtained when media were collected immediately postflow (not shown).

We also examined the effect of fluid flow on PGE_2 accumulation in DN-8 cells cultured at 48 h, a period after which DN-8 cells are as well coupled as MC3T3-E1 cells (Fig. 4*B*). Whereas exposure to fluid flow did not increase PGE_2 accumulation in media collected 1 h postflow from DN-8 cells cultured for 96 h, it did increase in media from DN-8 cells cultured for 48 h ($P < 0.005$ vs. no-flow controls). Similar results were obtained when media were collected immediately postflow (data not shown).

Ca^{2+} response to oscillatory fluid flow. In cells cultured for 96 h, there was a 7.9-fold increase ($P < 0.0007$

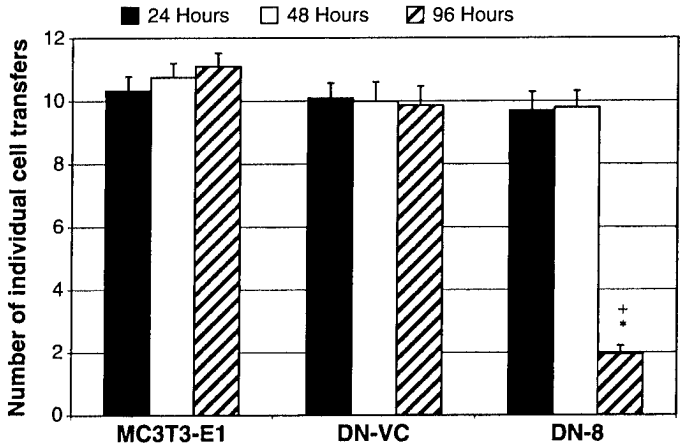

Fig. 3. Quantitative results of double-labeling assay at 24, 48, and 96 h in the 3 cell lines examined. MC3T3-E1 and DN-VC cell lines were highly coupled at all time points. No significant differences in coupling were found within or between these 2 cell lines at the various time points. The DN-8 cell line was well coupled at 24 and 48 h and not significantly different from the other cell lines at these time points. At 96 h, coupling in the DN-8 line was significantly diminished compared with the DN-8 line at 24 and 48 h ($P < 0.001$) as well as compared with the MC3T3-E1 and DN-VC lines at this time point ($P < 0.001$). Each bar is representative of a minimum of 60 cells (maximum 110) and is plotted as means ± SE with individual cell transfers not counted past a maximum of 15 cells. *Significantly different from 24- and 48-h time points within group; $^+$significantly different from 96-h time points in MC3T3-E1 and DN-VC cell lines.

vs. no flow) in the percentage of MC3T3-E1 cells responding to 3 min of oscillatory fluid flow with an increase in $[Ca^{2+}]_i$, an 8.9-fold increase in DN-VC cells ($P < 0.0001$), and a 9.3-fold increase in DN-8 cells ($P < 0.0003$; Fig. 5). The fold increases were not statistically different among the three cell lines. No significant differences in $[Ca^{2+}]_i$ amplitude within or among groups were observed (data not shown), a finding also made in our previous work with human fetal osteoblastic cells (41).

PGE_2 accumulation in the presence of thapsigargin. One interpretation of our findings that GJIC contributed to the PGE_2 but not the $[Ca^{2+}]_i$ response to fluid flow in DN-8 cells is that Ca_i^{2+} mobilization may not be critical to fluid flow-induced PGE_2 accumulation. To address this issue, we examined the effect of thapsigargin on fluid flow-induced PGE_2 accumulation. In the presence of thapsigargin, media from MC3T3-E1 and DN-VC cells collected 1 h postflow had a 92.1% and 278%, respectively, increase in PGE_2 accumulation relative to no-flow control. Once again, fluid flow did not increase PGE_2 accumulation in DN-8 cells cultured for 96 h and thus coupled poorly. Therefore, thapsigargin did not significantly alter the PGE_2 response to fluid flow in any of the cell lines examined (Fig. 6).

DISCUSSION

In this study, we set out to investigate the role of gap junctions and GJIC in mechanotransduction mechanisms in bone. We applied a novel dominant-negative genetic intervention strategy to MC3T3-E1 osteoblastic parent cells to render them communication deficient. We subjected the resulting cell line to oscillatory fluid flow and measured flow-induced PGE_2 release and changes in $[Ca^{2+}]_i$. This is the first study to examine GJIC in bone cell ensemble responsiveness to fluid flow, and, while only the second study to examine the effects of oscillatory fluid flow on $[Ca^{2+}]_i$ in osteoblastic cells, it is the first to quantify the PGE_2 response. We found that a breakdown in gap junction coupling had no effect on changes in $[Ca^{2+}]_i$ but resulted in a significant inhibition of oscillatory fluid flow-induced PGE_2 release, suggesting that gap junctions play a pivotal

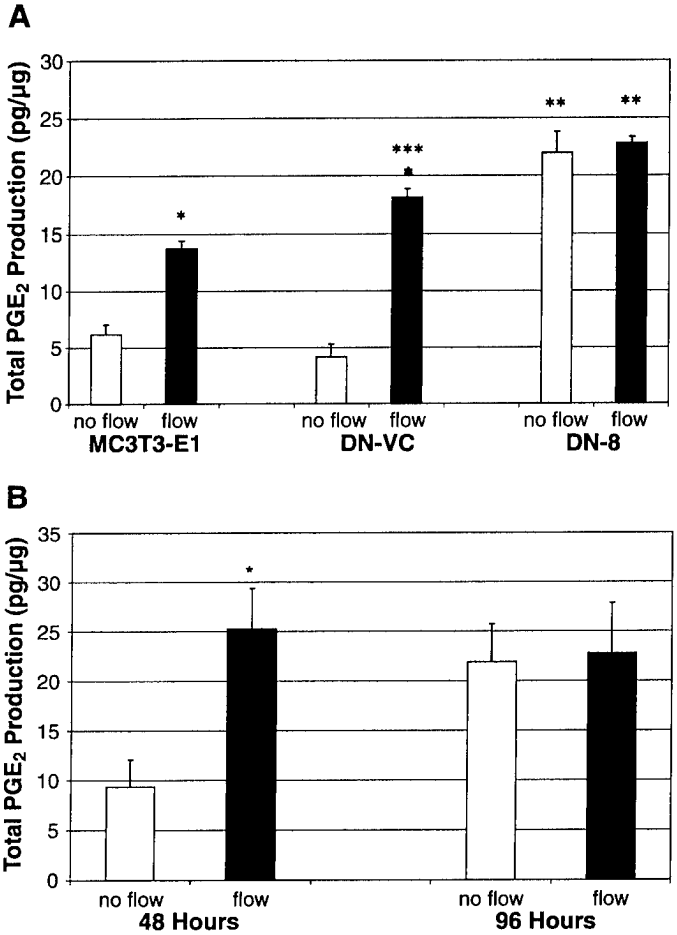

Fig. 4. Results of prostaglandin E_2 (PGE_2) quantification in the 3 cell lines examined. The numbers are representative of total PGE_2 accumulation in the media normalized to total protein and collected after 1-h incubation period. *A*: at 96 h, the MC3T3-E1 and DN-VC cell lines responded to fluid flow with an increase in PGE_2 accumulation, whereas the DN-8 cell line did not respond to fluid flow. Although results are shown for the 1-h collections only, similar trends were exhibited in the 0-h collections (data not shown). Interestingly, baseline levels were elevated in this line at this time point. *B*: at 48 h, the DN-8 cell line displayed an increase in PGE_2 accumulation in response to oscillatory fluid flow with more accumulation obtained from the 1-h postflow samples. At 96 h, the DN-8 cell line responded to flow with no significant increases in PGE_2 accumulation from either the 0-h or 1-h collections. All results shown are plotted as means ± SE with each value representative of at least 10 experiments. *Significantly different from no-flow control within group; **significantly different from media collected 1-h postflow at same time point in DN-VC and MC3T3-E1; ***significantly different from media collected 1-h postflow at same time point in MC3T3-E1.

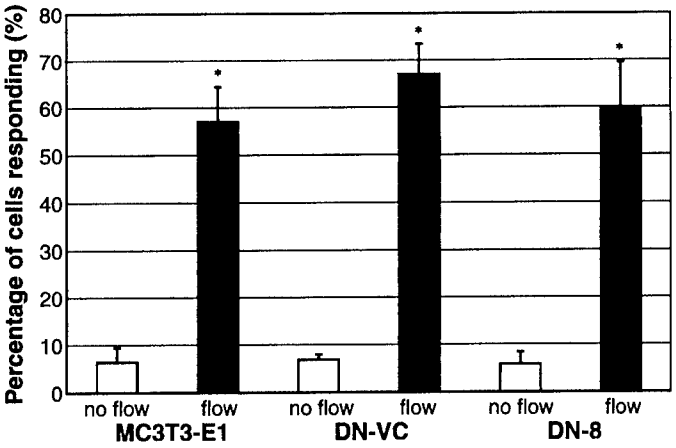

Fig. 5. Results of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) imaging at 96 h. In all cell lines examined, oscillatory fluid flow induced a significant increase in the percentage of cells responding with an increase in $[Ca^{2+}]_i$ ($P < 0.0003$, at least). Significant differences were not observed between the groups when comparing the no-flow controls or the flowed samples. All results are shown plotted as means ± SE with each value representative of at least 4 experiments (MC3T3-E1) or 6 experiments (DN-VC and DN-8). *Significantly different from no-flow control within group.

role in the mediation of oscillatory fluid flow-induced PGE_2 production in osteoblastic cells.

To verify the effectiveness of the dominant-negative strategy used to render the DN-8 cells communication deficient, we quantified the extent of coupling in the DN-8 cells at 24, 48, and 96 h in culture. These results were compared with coupling experiments conducted at the same time points in the communication-intact, control-transfectant DN-VC cell line. We found an 80.1% decrease in coupling in the DN-8 cells between 48 and 96 h in culture, whereas no significant change was noted in the DN-VC cells over the same time period. These results indicate that GJIC in only the DN-8 cell line was dependent on time in culture. Therefore, because the cells are genetically identical and cultured under the same culture conditions, these cells provide a novel model system in the analysis of GJIC in bone cell responsiveness to fluid flow.

To address the role that gap junctions play in the oscillatory fluid flow-induced PGE_2 response, we subjected the cell lines to 1 h of oscillatory fluid flow and measured PGE_2 accumulation in the media compared with PGE_2 accumulation in media from no-flow controls. At the 48-h time point, when intact GJIC was exhibited in the DN-8 cell line, the application of oscillatory fluid flow resulted in significant increases in PGE_2 accumulation. However, at the 96-h time point, when GJIC was inhibited, no increase in oscillatory fluid flow-induced PGE_2 accumulation resulted. In contrast, the DN-VC cell line responded at both time points with significant increases in PGE_2 accumulation. Thus we found that a breakdown in coupling was accompanied by a significant decrease in PGE_2 responsiveness to oscillatory fluid flow. These findings strongly suggest that gap junctions and GJIC are necessary in the signal transduction pathway whereby osteoblastic cells increase production of PGE_2 in response to oscillatory fluid flow and that a GJIC-dependent pathway exists.

To address the role that gap junctions play in the mediation of oscillatory fluid flow-induced changes in $[Ca^{2+}]_i$, we subjected the cell lines to 3 min of oscillatory fluid flow and measured $[Ca^{2+}]_i$ compared with $[Ca^{2+}]_i$ of no-flow controls. At the 96-h time point, DN-8 and DN-VC cells responded to oscillatory fluid flow with significant increases in $[Ca^{2+}]_i$. Moreover, differences in flow-induced $[Ca^{2+}]_i$ were not significantly different in the DN-8 and DN-VC lines at this time point. Thus we found that a breakdown in coupling was not accompanied by a significant change in $[Ca^{2+}]_i$ and that the Ca^{2+} responses of the cell lines, regardless of degree of coupling, were equally responsive. These findings strongly suggest that gap junctions and GJIC are not necessary in the signal transduction pathway whereby osteoblastic cells increase $[Ca^{2+}]_i$ in response to oscillatory fluid flow and that a GJIC-independent pathway exists.

In this study, we found that although coupling-deficient osteoblastic cells responded to the application of oscillatory fluid flow with significant increases in PGE_2 release, changes in $[Ca^{2+}]_i$ were not found due to changes in coupling. These findings suggest that the PGE_2 and $[Ca^{2+}]_i$ responses elicited via oscillatory fluid flow may be unlinked in these osteoblastic cells, a notion contradictory to prevailing opinion. To address this issue, we subjected the cell lines to oscillatory fluid flow in the presence of thapsigargin. We found that the introduction of thapsigargin did not significantly affect PGE_2 production, whereas the Ca^{2+} response was completely annihilated (data not shown). Furthermore, because we have data indicating that the only source of Ca^{2+} in the MC3T3-E1 cells is from intracellular stores (42) that are emptied by the thapsigargin, our findings provide concrete evidence to suggest a separation of pathways is involved in Ca^{2+} wave propagation and PGE_2 production in osteoblastic cells.

In this study, we set out to investigate the role of gap junctions in mediating oscillatory fluid flow-induced

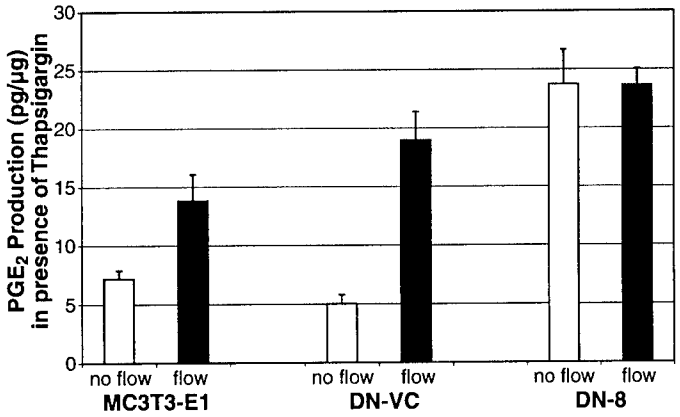

Fig. 6. Results of PGE_2 quantification in the presence of thapsigargin at 96 h in the 3 cell lines examined. At 96 h, the PGE_2 response of the cell lines to oscillatory fluid flow was not altered by the presence of thapsigargin. All results are shown plotted as means ± SE with each value representative of a minimum of 2 experiments.

PGE_2 response in osteoblastic cells. Inasmuch as this was our goal, we were largely interested in whether the application of oscillatory fluid flow resulted in significant increases in PGE_2 production in the cell lines. However, studies have shown that the exact PGE_2 time course has yet to be elucidated and that flow-induced PGE_2 production is not obliterated with the cessation of the stimulant (22). To address the time-dependent response of oscillatory fluid flow-induced PGE_2 release in these cell lines, PGE_2 accumulation in the media was measured at two time points after cessation of flow. In the first approach, the flowed media were collected immediately after flow exposure; in the second approach, the flowed cells were placed in an equivalent volume of fresh media immediately postflow and incubated for 1 h. We found that in the coupling-intact DN-VC cells, flow-induced levels of PGE_2 accumulation in media from cells incubated for 1 h postflow were significantly higher compared with media from cells collected immediately postflow. Similarly, PGE_2 levels in media from no-flow control cells incubated for 1 h postflow were significantly elevated compared with levels from no-flow control cells collected immediately postflow. These findings were similar to those observed in the DN-8 cell line at 48 h when coupling was still intact, suggesting that a comparison of baseline PGE_2 accumulation levels from media collected from incubated postflow cells is more appropriate than in media collected immediately postflow and may be more sensitive to extracellular regulation.

Curiously, we found that basal PGE_2 levels were elevated in the DN-8 cells at the 96-h time point. Although we are unable to definitively explain this result, it is unlikely that the elevation was a result of the transfection process, since the control transfectant DN-VC cells did not exhibit a similar trend. To further address this issue, we added the ionophore 4-bromocalcium (50 μM for 15 min) to confluent slides of DN-8 cells and measured PGE_2 accumulation levels in excess of those shown in Fig. 4 (data not shown), indicating that increases beyond these basal levels were indeed possible. In any case, we do not feel that these findings detract from the main finding of this paper, namely that GJIC-deficient cells do not respond to oscillatory fluid flow with an increase in PGE_2 release.

It is also possible that factors other than GJIC are involved in the responsiveness of a cell ensemble to oscillatory flow by affecting the inherent responsiveness of the individual cells. For instance, it is possible that membrane permeability or morphological changes in the membrane are important and could lead to sensitivity changes in protein receptors, ion channels, and cytoskeletal elements. This may help to explain the increase we observed in basal PGE_2 production levels in the DN-8 cells at the 96-h time point. Furthermore, it is also possible that GJIC may affect such changes in cellular sensitivity. For instance, it has previously been shown that GJIC contributes indirectly to morphological changes by contributing to extracellular matrix organization (3).

Although a substantial body of evidence exists linking GJIC and cellular responsiveness to physical stimuli, the work to date has provided only indirect evidence. For instance, several studies have shown that the application of physical stimuli in vitro results in increased Cx43 expression in both osteoblasts (43) and osteocytes (29), a finding that parallels those in smooth muscle (5) and endothelial cells (11). However, these studies do not address changes in coupling or that changes in coupling influences the sensitivity of the cell ensemble. Thus it is important to distinguish between connexin formation and functional coupling, which would provide direct evidence to suggest that gap junctions are important in mechanotransduction.

In this study, we investigated the role of GJIC in oscillatory fluid flow-induced PGE_2 production and changes in Ca_i^{2+} signaling. We found direct evidence to indicate that the PGE_2 response was dependent on gap junctions, demonstrated by the lack of PGE_2 released in the gap junction-deficient DN-8 cell line compared with the DN-VC cell line. In addition, by investigating real-time Ca_i^{2+} responses in these cell lines, we found that all three cell lines were able to respond to oscillatory fluid flow with an immediate increase in $[Ca^{2+}]_i$. Finally, by blocking the Ca_i^{2+} response with thapsigargin, we demonstrated that the PGE_2 response in MC3T3-E1 cells to oscillatory fluid flow does not depend on an increase in $[Ca^{2+}]_i$. Together, these findings strongly suggest an important role for gap junctions and GJIC in bone cell mechanotransduction mechanisms.

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P2Y PURINERGIC RECEPTORS ARE NECESSARY FOR OSCILLATORY FLUID FLOW INDUCED CALCIUM MOBILIZATION IN OSTEOBLASTIC CELLS

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INTRODUCTION

Lacunar-canalicular fluid flow has been demonstrated to be a potentially important physical signal for mechanical loading-induced changes in bone cell metabolism. Recently we reported that oscillatory fluid flow activated MC3T3-E1 osteoblastic cell intracellular calcium (Ca^{2+}_i) mobilization via the IP_3 pathway in the presence of 2% fetal bovine serum (FBS) (1). We and other investigators (2) also found that extracellular signaling molecules present in FBS are necessary for fluid flow induced increases in $[Ca^{2+}]_i$ in bone cells. However, the nature of these signaling molecules and their receptors are unknown. Previously it was demonstrated that extracellular nucleotides elevated $[Ca^{2+}]_i$ in osteoblastic cells (3). Thus, we hypothesized that extracellular nucleotides are involved in flow induced Ca^{2+}_i mobilization. The first goal of this study was to determine which signaling molecules within FBS might contribute to fluid flow induced increases in $[Ca^{2+}]_i$ in bone cells. The second goal was to determine the specific receptors of these molecules in osteoblastic MC3T3-E1 cells.

METHODS

Cell culture and fluid flow chamber: The mouse osteoblastic cell line MC3T3-E1 was cultured in α-MEM containing 10% FBS and the rat osteoblastic cell line ROS 17/2.8 (ROS), which does not normally express P2Y2 receptors, was grown in MEM supplemented with 10% heat-inactivated calf serum. ROS cells transfected with human P2Y2 purinergic receptor (ROS/P2U) were cultured in the presence of 400 μg/ml Geneticin (4). All cells were maintained in 5% CO_2 at 37°C. The fluid flow chamber employed in this study is a parallel plate design. A flow delivery device generated 1Hz sinusoidaly oscillating flow (peak shear stress 2N/m^2). Calcium imaging: Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was quantified using Fura-2 AM. Basal $[Ca^{2+}]_i$ was sampled for 3 min followed by 3 min of oscillating flow. Control bathing media consisted of α-MEM (or MEM) medium with/without 2% FBS. Image acquisition and analysis software was used to capture and convert fluorescent signals into real-time $[Ca^{2+}]_i$ values. A cell was considered responsive if the peak increase in $[Ca^{2+}]_i$ following the oscillating flow period was at least two-fold greater than that of the average baseline level. Oligonucleotide treatment: Antisense oligonucleotides P2Y2-AS 5'-CAG GTC TGC TGC CAT-3' and the scrambled oligonucleotides P2Y2-SC 5'-GTG CTC GTA CGT ACC-3' were used to treat the cells. $8x10^4$ MC3T3-E1 cells pre-cultured for 24 hr were incubated for 2.5 hr with a fresh culture medium containing oligonucleotides (0.5μg). After oligonucleotide treatment, cells were cultured in a fresh culture medium for another 24 hr to perform fluid flow experiments. Reagents: Five nucleotides (ATP, UTP, GTP, CTP and TTP) were employed as extracellular signaling molecules to test in flow experiments. Apyrase (10U/ml), an enzyme that rapidly hydrolyses 5' nucleotide-triphosphates to monosphophates, was used to block these nucleotides effects. Different purinoceptor agonists (ADP, UDP, ATPγS, and Adenosine) and antagonist (PPADS) were applied to identify specific purinoceptors.

RESULTS

First, we found that oscillatory fluid flow (±2N/m^2) in the absence of extracellular signaling molecules (i.e. no FBS in medium) failed to increase $[Ca^{2+}]_i$ in MC3T3-E1 cells (Fig. 1A). Apyrase (10U/ml) prevented the fluid flow induced increases in $[Ca^{2+}]_i$ in the presence of FBS. Two of the five nucleotide-triphosphates, ATP and UTP (5μM), were able to restore the ability of fluid flow to increase $[Ca^{2+}]_i$ in the absence FBS. Secondly, to identify the specific receptors responsible for the flow effect, we added purinoceptor agonists ADP or UDP, neither of which was able to increase $[Ca^{2+}]_i$ in the absence of FBS. Adenosine, a P1 receptor agonist, and ATPγS, a P2X and P2Y11 receptor agonist, added to medium did not restore the ability of fluid flow to increase $[Ca^{2+}]_i$. PPADS, a P2X antagonist, did not have any effect on the Ca^{2+}_i response in the presence of FBS. However, Pertussis Toxin (PTX) which inhibits the $G_{i/o}$ protein, to which P2Y receptors, but not other purinoceptors, are coupled, inhibited fluid flow induced increases in $[Ca^{2+}]_i$. Additionally, P2Y2 antisense oligonucleotide treatment decreased the percentage of cells responding to flow with an increase in $[Ca^{2+}]_i$, relative to scrambled P2Y2 oligonucleotide treatment (29.0% vs. 65.3% respectively) in the presence of 2% FBS. Finally, only 25.8% of ROS 17/2.8 cells lacking P2Y2 receptors responded with an increase in $[Ca^{2+}]_i$ whereas 69.0% of ROS cells transfected with P2Y2 receptor cDNA responded.

DISCUSSION

We have shown that oscillatory fluid flow did not activate Ca^{2+}_i mobilization in the absence of FBS while apyrase completely blocked the flow induced $[Ca^{2+}]_i$ increases in the presence of FBS. In addition, ATP/UTP restored the ability of fluid flow to increase $[Ca^{2+}]_i$ suggesting that ATP or UTP may be mediating the effect of fluid flow on $[Ca^{2+}]_i$. Neither ADP, UDP nor their receptors, P2Y1 and P2Y6, were involved in the fluid flow response. Adenosine, ATPγS, PPADS, and PTX data suggest that P1 purinoceptors, P2X and P2Y11 receptors were also not involved in flow induced increase $[Ca^{2+}]_i$. Thus, by process of elimination, our data suggest that P2Y receptors (P2Y2 or P2Y4) are involved in the Ca^{2+}_i response to fluid flow. Furthermore, antisense treatment and ROS/P2U data confirm that P2Y2 receptors play a major role in oscillatory fluid flow induced Ca^{2+}_i mobilization in osteoblastic cells. Taken together, these data suggest that oscillatory fluid flow acts through the interaction of extracellular nucleotide ATP/UTP with P2Y (P2Y2, or P2Y4) purinoceptors to induce Ca^{2+}_i mobilization in osteoblastic cells. Our finding is the first direct experimental evidence showing that fluid flow and the extracellular signaling molecules ATP/UTP with their specific P2Y receptors are essential regulators for bone cell mechanotransduction.

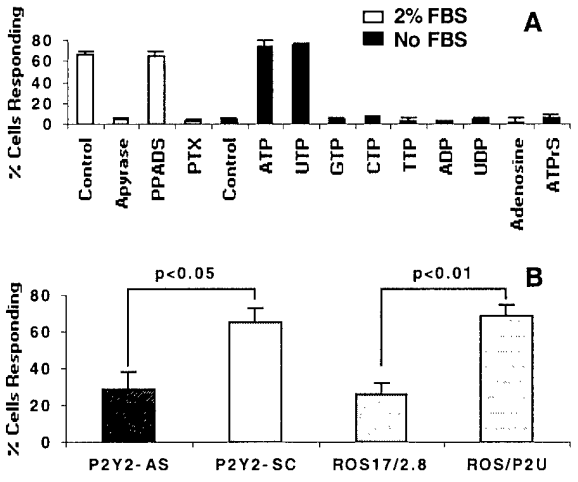

Figure 1

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Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent

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Donahue, Seth W., Christopher R. Jacobs, and Henry J. Donahue. Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent. *Am J Physiol Cell Physiol* 281: C1635–C1641, 2001.—Bone adaptation to mechanical loading is dependent on age and the frequency and magnitude of loading. It is believed that load-induced fluid flow in the porous spaces of bone is an important signal that influences bone cell metabolism and bone adaptation. We used fluid flow-induced shear stress as a mechanical stimulus to study intracellular calcium (Ca_i^{2+}) signaling in rat osteoblastic cells (ROB) isolated from young, mature, and old animals. Fluid flow produced higher magnitude and more abundant $[Ca^{2+}]_i$ oscillations than spontaneous oscillations, suggesting that flow-induced Ca_i^{2+} signaling encodes a different cellular message than spontaneous oscillations. ROB from old rats showed less basal $[Ca^{2+}]_i$ activity and were less responsive to fluid flow. Cells were more responsive to 0.2 Hz than to 1 or 2 Hz and to 2 Pa than to 1 Pa. These data suggest that the frequency and magnitude of mechanical loading may be encoded by the percentage of cells displaying $[Ca^{2+}]_i$ oscillations but that the ability to transduce this information may be altered with age.

mechanotransduction; osteoblast; calcium signaling; bone adaptation

BONES ADAPT to mechanical loading. When normal mechanical loading is absent, bone mass is removed. For example, disuse osteopenia occurs in the tibias of astronauts who experience microgravity (49), in patients confined to prolonged bedrest (28), in immobilized bones following surgery (30), and in patients with total arthroplasty (29). When habitual bone loading is exceeded, bone mass is added. For instance, periosteal and endosteal bone areas have been found to be significantly higher in the dominant arm of tennis players (3). Bone mineral density and cross-sectional moment of inertia have been found to be significantly higher in the dominant humeri of tennis players, regardless of the age at which they started playing (16). However, the effect of mechanical loading on bone mass was more than twofold greater in young players than in players who began playing after reaching skeletal maturity. These data suggest that growing bones are more adaptable to mechanical loading than adult bones. Turner et al. (44–46) have demonstrated the ability of rat long bones to adapt to unaccustomed mechanical loading. They showed that new bone formation in tibias loaded in four-point bending was dependent on the frequency and magnitude of loading (44, 45). They also demonstrated that the ability of long bones to adapt to mechanical loading was diminished in 19-mo-old rats compared with 9-mo-old rats (46). At the highest bending load, the relative bone formation rate was more than 16-fold lower in the older rats. These findings parallel human studies, which suggest that cells in growing bones are more sensitive to mechanical signals than cells in adult bones.

It is believed that physical activities, which produce bending loads in bones, induce fluid flow in the porous spaces of bone (10, 15, 26, 27, 50). This fluid flow is believed to be an important physical signal that influences bone cell metabolism and bone adaptation to mechanical loading (7, 15, 27). Bone cells produce adaptations to mechanical loading: osteoblasts add bone mass when loading becomes excessive, and osteoclasts remove unneeded bone. However, the biochemical signaling pathways that mediate bone adaptation to mechanical loading are unknown. Understanding how individual bone cells respond to mechanical stimuli with biochemical responses and how these responses change with age may help elucidate our understanding of mechanically induced bone adaptations and the etiology of bone diseases such as osteoporosis.

In vitro, physical stimuli activate numerous signaling molecules in bone cells, including intracellular calcium (9, 17, 18, 51, 52, 55), prostaglandins (1, 36, 40), inositol trisphosphate (36), and nitric oxide (25, 32, 40). Mechanical stimuli also have been shown to upregu-

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late gene expression in bone cells (9, 20, 54). Intracellular calcium oscillations are important signaling mechanisms for many cellular processes (e.g., differentiation, proliferation, and gene transcription) (5). Calcium signaling also is an early response in bone cell mechanotransduction and can influence downstream signaling events. For example, blocking mechanically induced intracellular calcium oscillations also blocks gene expression (9, 54), prostaglandin release (1), and cytoskeletal reorganization (9).

In vivo rat studies have demonstrated that long bone adaptation to mechanical loading is magnitude and frequency dependent and that the capacity for adaptation decreases with age (44–46). Therefore, it is likely that biochemical signaling in bone cells in response to mechanical stimuli is also dependent on age and on the magnitude and frequency of the physical signal. We hypothesized that fluid flow-induced oscillations in cytosolic calcium concentration ($[Ca^{2+}]_i$), in osteoblastic cells isolated from rat long bones, would be dependent on loading frequency, shear stress magnitude, and age of the rat from which the cells were isolated. Biochemical messages encoded by $[Ca^{2+}]_i$ oscillations may be determined by the magnitude and/or frequency of the oscillation (6, 43). It also is thought that calcium signaling requires coordinated Ca_i^{2+} signaling events in cell ensembles.(21) Therefore, we chose the percentage of cells displaying $[Ca^{2+}]_i$ oscillations and the magnitude of the oscillations as the independent response variables to fluid flow-induced shear stress.

METHODS

Bone cells. Rat osteoblastic cells (ROB) were isolated from the humeri, tibias, and femora of young (4 mo, $n = 7$), mature (12 mo, $n = 7$), and old (24 mo, $n = 7$) male Fisher 344 rats. All procedures were approved by the Institutional Animal Care and Use Committee at the M. S. Hershey Medical Center. The rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL) with a dosage of 50 mg/kg of bodyweight and were euthanized by exsanguination. The bones were extracted from the animals, and subperiosteal ROB were obtained by removing all soft tissues, including cartilage and periosteum, from the bones and performing sequential collagenase (Worthington Biochemical, Lakewood, NJ) digestions at 37°C. Cells from the first digestion were collected by centrifugation and discarded to eliminate any residual non-bone cells that were not removed by dissection. Cells from the second digestion were collected by centrifugation and grown to confluency in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Gaithersburg, MD), 20% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Cells from all six bones were pooled and grown to confluency in the same culture flask. We have shown previously that ROB isolated by this technique display characteristics of the osteoblast phenotype (13). We also stained ROB for alkaline phosphatase activity and performed a dye transfer assay to demonstrate gap junctional intercellular communication (53).

Three days before experimentation, the cells were plated on quartz microscope slides (76 × 26 × 1.6 mm) at a density of 75,000 cells per slide; cells were ~70% confluent on the day of experimentation. The cells were incubated at 37°C with 10 μM fura 2-AM (Molecular Probes, Eugene, OR) for 30 min before mechanical stimulation.

Fluid flow system. After fura 2 loading, the cell-seeded microscope slides were mounted in a parallel-plate flow chamber that was fixed to the stage of a fluorescent microscope. A fresh bolus of flow medium was added to the chamber, and the cells were left undisturbed for 30 min. The flow medium consisted of DMEM and 2% FBS. We have described previously the fluid flow system in detail (18); it will be described briefly here. To generate fluid flow-induced shear stresses on the cells in the chamber, a material-testing machine was used to pump a syringe, which was in series with rigid wall tubing and a flowmeter (Transonic Systems, Ithaca, NY), driving fluid through the chamber. This system produces laminar fluid flow in the chamber with an oscillating flow profile. Shear stresses on the chamber walls are proportional to the chamber dimensions and the rate of fluid flow (17). Thus we were able to generate shear stresses on the cells with magnitudes that they are predicted to experience in vivo (50).

Oscillating fluid flow was used because it more closely simulates physiological bone loading than steady or pulsatile flow (18). During experimentation, the cells were exposed to 3 min of oscillating fluid flow that produced shear stresses of 1 or 2 Pa at frequencies of 0.2, 1, or 2 Hz. Six slides of cells from each rat were randomly assigned to one of the six shear stress/frequency combinations.

Calcium imaging. Real-time $[Ca^{2+}]_i$ was quantified by using ratiometric dye methodology. When fura 2 binds Ca^{2+}, its maximal absorption wavelength shifts from 363 nm for Ca^{2+}-free fura 2 to 335 nm for Ca^{2+}-bound fura 2 (41). In practice, wavelengths of 340 and 380 nm are used for ratiometric measurements. The emission peak is near 510 nm for both Ca^{2+}-free and Ca^{2+}-bound fura 2. ROB ensembles were illuminated at wavelengths of 340 and 380 nm, emitted light was passed through a 510-nm filter, and images were collected with a charge-coupled device camera. Images of fluorescence intensities were collected every 2 s for a 3-min no-flow period (baseline) and for 3 min of oscillating fluid flow. $[Ca^{2+}]_i$ was determined from the ratio of the two emission intensities by using calibrated standards and image analysis software (Metaflour, West Chester, PA). Temporal $[Ca^{2+}]_i$ profiles were determined for 25–35 individual cells for each slide (Fig. 1).

Resting $[Ca^{2+}]_i$ was typically ≤50 nM in ROB. We defined a responsive cell as one that displayed a transient increase in $[Ca^{2+}]_i$ of at least 50 nM, because this represented at least a 100% increase over baseline. A numerical method known as Rainflow cycle counting was used to determine the magnitude of the calcium oscillations (19). We assessed the percentage of cells responding with a calcium oscillation and the magnitude of the responses.

Statistics. A factorial ANOVA was used to assess the influence of age, loading frequency, and shear stress on the percentage of cells responding to fluid flow with calcium oscillations and on the magnitude of the oscillations. ANOVAs were followed by Tukey's test for multiple mean comparisons. One-way ANOVAs were used to look for age-related differences for each shear stress/frequency combination. Use of ANOVA models requires the error terms to be normally distributed and requires constant variance for all factor levels (33). Studentized residuals were used to diagnose the validity of the model's assumptions. Frequency distributions of the residuals were used to check for outliers and normality of error terms. Plots of the residuals against fitted values were used to assess constancy of variance. Paired t-tests were used to compare the magnitudes of spon-

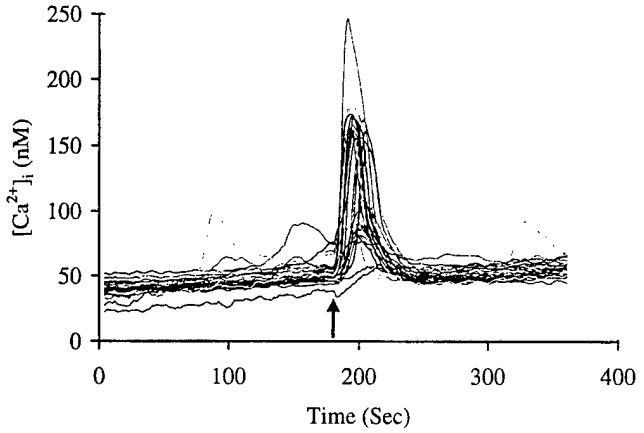

Fig. 1. Representative cytosolic calcium concentration ($[Ca^{2+}]_i$) profiles of 35 individual cells for a 3-min baseline period and 3 min of exposure to oscillating fluid flow [2 Pa, 1 Hz; rat osteoblastic cells (ROB) from a young rat]. Arrow indicates when flow was initiated. There were spontaneous $[Ca^{2+}]_i$ oscillations in the baseline period, and with the onset of fluid flow there was a greater and more coordinated response.

taneous and fluid flow-induced $[Ca^{2+}]_i$ oscillations in cells that had responses in both periods. A significance level of 0.05 was used for all statistical analyses.

RESULTS

ROB from all three age groups displayed abundant alkaline phosphatase staining in confluent cultures. Our laboratory has demonstrated previously (12) that confluent cultures of ROB from young, mature, and old animals display highly functional gap-junctional intercellular communication. We found comparable functional communication in subconfluent ROB, from all three age groups, that were seeded on quartz microscope slides (not shown). These data provide further verification that ROB display characteristics of the osteoblast phenotype.

Individual ROB demonstrated one of four $[Ca^{2+}]_i$ profiles over the 6-min imaging period: *1*) spontaneous oscillation in the baseline period and no oscillation in the flow period, *2*) oscillations in both the baseline and flow periods, *3*) no oscillation in the baseline period and an oscillation in the flow period, and *4*) no oscillations in either period (Fig. 1). Rarely, cells displayed multiple oscillations in the baseline or flow periods. For the cells that did so, peak oscillations were used for statistical analyses. $[Ca^{2+}]_i$ oscillations in both periods typically lasted 60 s and returned to near-baseline values.

There were spontaneous $[Ca^{2+}]_i$ oscillations of at least 50 nM in ROB from all three age groups during the no-flow period. Of all the young ROB that were analyzed, 10% displayed spontaneous $[Ca^{2+}]_i$ oscillations. Significantly ($P = 0.04$), fewer ROB from old rats displayed spontaneous $[Ca^{2+}]_i$ oscillations during the no-flow period (Fig. 2*A*). However, there were no significant ($P = 0.17$) differences in the magnitude of the $[Ca^{2+}]_i$ oscillations among the three age groups (Fig. 2*B*).

With the onset of fluid flow, there were immediate and transient increases in $[Ca^{2+}]_i$ that lasted ~60 s (Fig. 1). Peak values were reached ~15 s after the onset of fluid flow. Significantly ($P < 0.0001$), more cells displayed $[Ca^{2+}]_i$ oscillations during the fluid-flow period than during the baseline period. Age ($P = 0.008$), loading frequency ($P = 0.0001$), and shear stress ($P = 0.035$) significantly influenced the percentage of cells responding to fluid flow. Mature ROB were more responsive than old ROB (Fig. 3). Cells were more responsive to 0.2 Hz than to 1 or 2 Hz (Fig. 4) and to 2 Pa than 1 Pa (Fig. 5). However, the magnitude of fluid flow-induced $[Ca^{2+}]_i$ oscillations was not significantly ($P = 0.367$) affected by age, loading frequency, or shear stress magnitude. The magnitude (mean ± SD) of the fluid flow-induced $[Ca^{2+}]_i$ oscillations, for all six loading regimes pooled, were 113 ± 60 nM for young ROB, 139 ± 102 nM for mature ROB, and 116 ± 85 nM for old ROB.

When the subpopulation of cells that displayed $[Ca^{2+}]_i$ oscillations in both the baseline and fluid-flow periods were considered, the magnitude of the fluid flow-induced $[Ca^{2+}]_i$ oscillations were significantly ($P < 0.0004$) larger than the magnitude of the sponta-

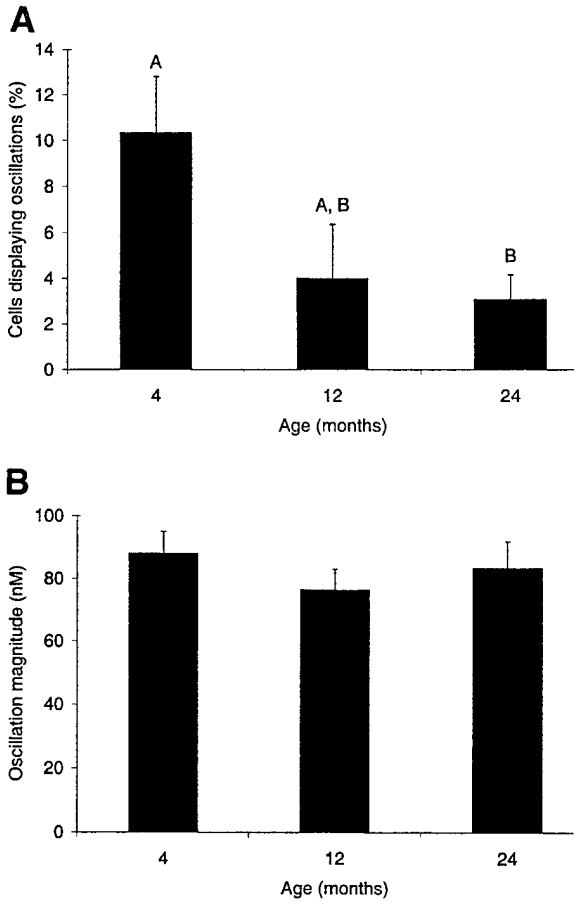

Fig. 2. *A*: influence of age on the percentage of cells displaying spontaneous $[Ca^{2+}]_i$ oscillations during the baseline period. Values are means with SE bars ($n = 42$ slides for each age group). Groups with the same letter (A, B) were not significantly different from each other. Cells from young rats showed significantly more oscillations than cells from old rats. *B*: magnitudes of spontaneous $[Ca^{2+}]_i$ oscillations in the baseline period were not significantly different among age groups.

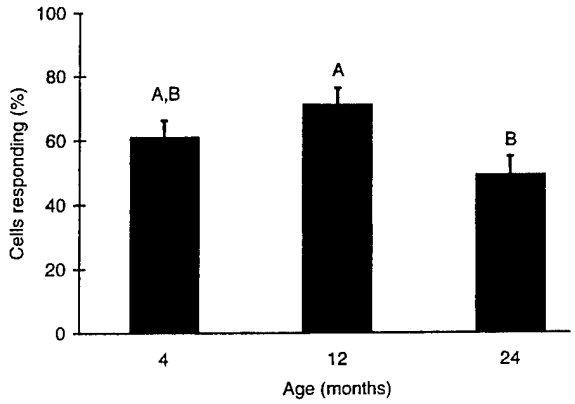

Fig. 3. Influence of age on the percentage of cells displaying $[Ca^{2+}]_i$ oscillations during the fluid-flow period. Values are means of all 6 loading regimes with SE bars (n = 42 slides for each age group). Groups with the same letter (A, B) were not significantly different from each other. A significantly higher percentage of cells from mature rats responded to fluid flow than cells from old rats.

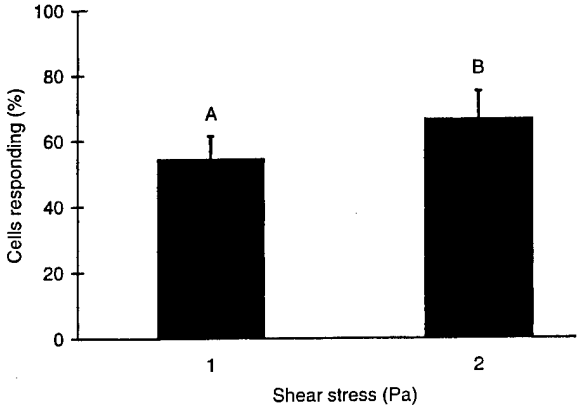

Fig. 5. Influence of shear stress on the percentage of cells displaying $[Ca^{2+}]_i$ oscillations during the fluid-flow period. Values are means of all age groups with SE bars (n = 63 slides for each shear stress). Shear stress of 2 Pa was significantly more stimulatory than shear stress of 1 Pa.

neous $[Ca^{2+}]_i$ oscillations for all three age groups. In this subpopulation, the magnitude of the fluid flow-induced $[Ca^{2+}]_i$ oscillations were 58% greater than spontaneous $[Ca^{2+}]_i$ oscillations in young ROB, 134% higher in mature ROB, and 81% higher in old ROB. Of the cells that displayed spontaneous $[Ca^{2+}]_i$ oscillations in the baseline period, 80% of young ROB, 75% of mature ROB, and 76% of old ROB also exhibited flow-induced oscillations. Fluid flow was even able to potentiate the magnitude of the $[Ca^{2+}]_i$ oscillation in cells that were displaying spontaneous oscillations at the onset of fluid flow (Fig. 6).

The age of the rat from which cells were isolated significantly affected the percentage of cells responding to fluid flow in the factorial model. A significantly (P = 0.008) larger percentage of ROB from mature rats (71%) had $[Ca^{2+}]_i$ oscillations than did ROB from old rats (49%) (Fig. 3). However, when each loading regime was considered separately, age did not significantly ($P > 0.141$) influence the percentage of cells responding to fluid flow at either 1 Pa (Fig. 7*A*) or 2 Pa (Fig. 7*B*).

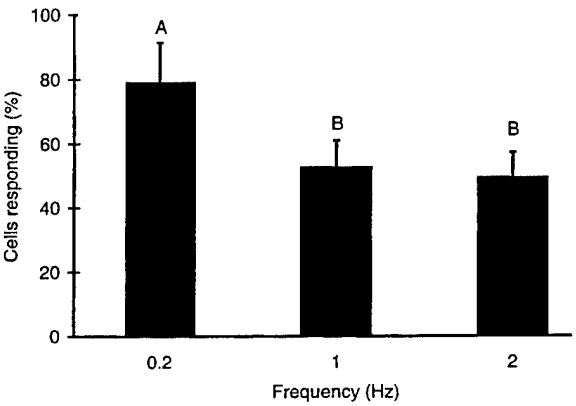

Fig. 4. Influence of loading frequency on the percentage of cells displaying $[Ca^{2+}]_i$ oscillations during the fluid-flow period. Values are means of all age groups with SE bars (n = 42 slides for each frequency). A frequency of 0.2 Hz was significantly more stimulatory than a frequency of 1 or 2 Hz.

For each shear stress/frequency combination, however, old ROB were always the least responsive.

DISCUSSION

Bones adapt to mechanical loading in a frequency- and magnitude-dependent fashion (44, 45). However, the bones of mature rats adapt better to unaccustomed mechanical loading than do the bones of old rats (46). It is widely believed that bone cells mediate bone adaptations to mechanical loading by activating signaling pathways that regulate bone modeling and remodeling (7, 8, 11, 15). We found that Ca_i^{2+} signaling in ensembles of osteoblastic cells was dependent on the frequency and magnitude of a mechanical stimulus. In addition, we found that ensembles of ROB from mature rats were more responsive to fluid flow than were ROB from old rats.

$[Ca^{2+}]_i$ oscillations are involved in many normal cellular processes such as proliferation and gene ex-

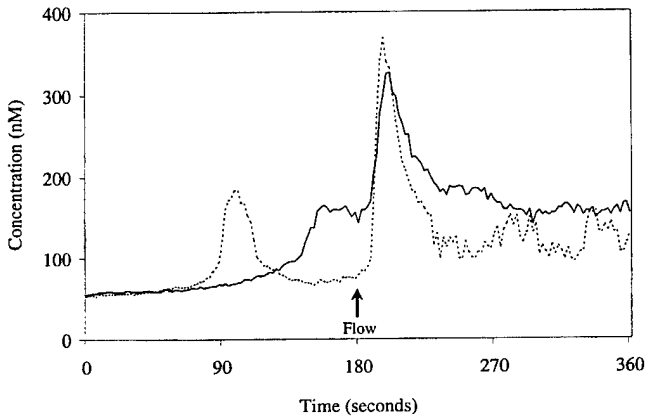

Fig. 6. $[Ca^{2+}]_i$ profiles of 2 individual cells that displayed $[Ca^{2+}]_i$ oscillations in both the baseline and flow periods. Fluid flow was able to induce larger magnitude $[Ca^{2+}]_i$ oscillations in cells that displayed spontaneous oscillations and returned to basal levels before the onset of flow (dashed line) and in cells that had not returned to basal levels before the onset of flow (solid line).

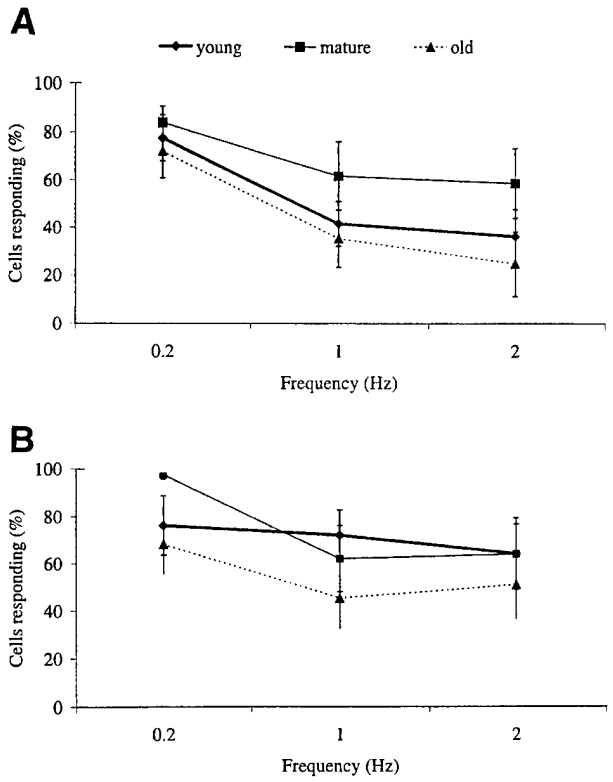

Fig. 7. Influence of age on the percentage of cells displaying $[Ca^{2+}]_i$ oscillations for each flow regime. Values are means with SE bars (n = 7 slides for data point). When each loading regime was considered independently, there were no significant differences among age groups at either 1 (*A*) or 2 Pa (*B*).

pression (5). The spontaneous oscillations that occurred in ROB during the baseline period may have been manifestations of normal cell cycle processes. We found that the number of spontaneous $[Ca^{2+}]_i$ oscillations declined with age in ROB, suggesting that bone cells from old animals are less metabolically active than cells from younger animals. To our knowledge, this is the first demonstration of an age-related decrease in basal Ca_i^{2+} signaling activity in any cell type. Clearly, mechanical stimulation produced a much more abundant and synchronized pattern of Ca_i^{2+} signaling than what occurred in the baseline period. Moreover, the magnitude of the flow-induced responses was significantly larger than the magnitude of the spontaneous oscillations. However, age, loading frequency, and shear stress influenced only the percentage of cells responding to fluid flow; they did not affect the magnitude of the $[Ca^{2+}]_i$ oscillations. These findings suggest that the percentage of cells responding with $[Ca^{2+}]_i$ oscillations may encode physical stimulus information. Indeed, the intercellular propagation of Ca_i^{2+} waves is a mechanism for many cell types to coordinate their activities (21). It also is believed that a threshold of $[Ca^{2+}]_i$ is required to activate a signaling cascade (43). In light of these views, it is reasonable to hypothesize that the abundant higher magnitude $[Ca^{2+}]_i$ oscillations caused by fluid flow encode a different biochemical message than the sparse lower magnitude $[Ca^{2+}]_i$ oscillations that occurred spontaneously during the baseline period.

Aging is known to impair osteoblast differentiation and activity, bone formation, and the material properties of bone (23, 31, 34, 37). Furthermore, aging impairs agonist-stimulated activity of second messengers such as cAMP and Ca^{2+} (14, 24), and Ca_i^{2+} signaling has been linked to the differentiation of cells from the mesenchymal lineage (4). We found that the percentage of cells displaying spontaneous $[Ca^{2+}]_i$ oscillations declined with age; significantly more ROB from young rats displayed spontaneous $[Ca^{2+}]_i$ oscillations than did ROB from old rats. There were also age-related differences in fluid flow-induced $[Ca^{2+}]_i$ oscillations, although in a more complex fashion. Significantly more ROB from mature rats displayed fluid flow-induced $[Ca^{2+}]_i$ oscillations than did ROB from old rats, but there were no differences between young and old ROB. These differences in the age-related trends of basal and fluid flow-induced Ca_i^{2+} signaling are difficult to interpret. Indeed, one would expect that ROB from rapidly growing young rats would display greater responses than ROB from old rats with slower growing bones. However, age-related differences in bone adaptation to unaccustomed mechanical loading has not been evaluated in the three age groups studied here. Although it has been demonstrated that 9-mo-old rats adapt better to mechanical loading than do 19-mo-old rats, it is unclear how the mechanical adaptations of young animals compare with those of mature and old animals (46).

Comparisons with in vivo data are further complicated when the influence of frequency and shear stress on Ca_i^{2+} signaling is examined in ROB. In vivo, bone formation increases when the frequency and magnitude of the mechanical stimulus increases (44, 45). We found contradicting results with $[Ca^{2+}]_i$ oscillations in ROB: the percentage of cells responding increased with increased shear stress but decreased with increased frequency. Clearly there is not a simple relationship between Ca_i^{2+} signaling and bone adaptation. In fact, it is likely that bone adaptation to mechanical loading involves the complex interactions of several mechanotransduction signaling pathways (35). Mounting in vitro mechanotransduction data supports a role for Ca_i^{2+} signaling in bone adaptation to mechanical loading (1, 54). For example, when mechanically induced calcium signaling in bone cells is inhibited by calcium channel blockers, mRNA expression of an abundant bone matrix protein (osteopontin) and the release of a potent stimulator of bone formation (prostaglandin E_2) are also inhibited (1, 54). Moreover, calcium channel blockers prevent mechanical loading-induced prostaglandin release in bone organ culture (35). A well-defined role for Ca_i^{2+} signaling in the bone adaptation, mechanotransduction signaling pathway has yet to be elucidated.

Primary bone cell cultures are necessary to study the effects of aging. However, a limitation of ROB cultures is that they likely contain a heterogeneous population of cells derived from bone. We cannot rule out the

possibility that the cultures contain nonosteoblastic or preosteoblastic populations. However, the ROB display an osteoblastic morphology and express phenotypic markers of osteoblasts (i.e., alkaline phosphatase, type I collagen, osteopontin, and parathyroid hormone receptor) (14, 24). It is possible that age-related differences in the degree of cellular heterogeneity of our cell populations contributed to differences in responsiveness to fluid flow. Indeed, this also may be the case in vivo.

There were no differences in the percentage of ROB responding between loading frequencies of 1 and 2 Hz, but a significantly larger percentage of ROB responded to 0.2 Hz. One possible explanation for these findings is cellular viscoelasticity. Because cells are viscoelastic, they may be less stiff and more deformable at lower loading rates. Thus it is possible that the mechanotransducing "machinery" (e.g., stretch-activated ion channels, cell surface receptors, cytoskeleton, etc.) is more likely to be activated at lower loading rates. However, the possibility that the results can be explained, at least in part, by molecular transport phenomena cannot be overlooked. It is well known that serum constituents (e.g., ATP) can function as agonists for $[Ca^{2+}]_i$ oscillations in a concentration-dependent fashion (39, 42, 43). Lower loading frequencies require the mechanical loading apparatus to pump larger volumes of medium, and thus more serum constituents, through the flow chamber to maintain shear stress levels. Therefore, the larger percentage of ROB responding to lower frequency loading may have resulted from larger agonist volumes flowing through the chamber. Similarly, this may explain why 2 Pa were significantly more stimulatory than 1 Pa; the development of higher shear stresses on the flow chamber walls, for a given frequency, requires larger volumes of fluid flow. However, it recently has been shown that shear stress in the absence of serum can stimulate a Ca_i^{2+} signaling in bone cells, but the response is enhanced by mechanical stimulation in the presence of serum (2). Furthermore, it has been shown that parathyroid hormone modulates Ca_i^{2+} signaling in bone cells, suggesting that bone cells may be sensitized to physical stimuli by biomolecules (38). These findings suggest that appropriate levels of both mechanical loading and biochemical constituents are required to mediate cellular mechanotransduction and bone adaptation to mechanical loading.

It has been postulated that gap junctions play a role in communicating mechanical signals in bone cell ensembles (11). In fact, a diverse array of extracellular stimuli (e.g., hormonal, electrical, and mechanical) has been shown to influence gap-junctional intercellular communication, which often involves the propagation of intercellular $[Ca^{2+}]_i$ oscillations (12, 22, 47, 48). However, intercellular $[Ca^{2+}]_i$ oscillations in bone cells also can be propagated by ATP activation of P2Y receptors (22). Because we found no age-related changes in gap-junctional communication in ROB, we postulate that the age-related decrease in the number of cells displaying mechanically induced $[Ca^{2+}]_i$ oscillations involves a defect in the ATP/P2Y mechanism.

In summary, we found that fluid flow-induced Ca_i^{2+} signaling in osteoblastic cells is age, frequency, and magnitude dependent. Cells from young rats showed more basal $[Ca^{2+}]_i$ activity than did old ROB, and mature ROB were more responsive to fluid flow than were old ROB. Low frequency and high shear stress loading regimes were the most stimulatory. We also showed that fluid flow produced higher magnitude and more abundant $[Ca^{2+}]_i$ oscillations than spontaneous oscillations. Ultimately, understanding mechanotransduction pathways in bone cells and how they are influenced by age and mechanical loading parameters may help elucidate the etiologies of bone diseases such as senile and disuse osteoporoses.

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REFRACTORY PERIOD OF FLUID FLOW INDUCED CALCIUM SIGNALING IN OSTEOBLASTIC CELLS

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Introduction

Partitioning a daily mechanical stimulus into discrete loading bouts enhances bone formation in rat tibiae.[1] This finding suggests that the bone cells involved in mediating mechanically induced bone adaptation have a refractory period, during which they are insensitive to additional mechanical stimuli. Mechanically induced fluid flow in bone may contribute to adaptation by providing cells with physical stimulation and enhancing molecular transport. Intracellular calcium is believed to be an important signaling molecule for bone cell mechanotransduction because it has been shown to influence mechanically induced prostaglandin release and gene expression.[2, 3] We hypothesized that osteoblastic cells have a refractory period, during which cytosolic calcium oscillations are insensitive to additional bouts of oscillating fluid flow.

Methods

Bone Cells Subperiosteal osteoblastic cells were isolated from the humeri, tibiae, and femora of male Fisher 344 rats. All soft tissues were stripped from the bones and cells were isolated by sequential collagenase digestions at 37°C. Cells from the second digestion were collected by centrifugation and grown to confluency in DMEM, 20% FBS, and 1% penicillin/streptomycin. Cells isolated by this technique display osteoblastic characteristics.[4] Cells were plated on quartz microscope slides at concentrations that reached 70% confluency on the day of experimentation. Cells were incubated at 37°C with 10 μM of the fluorescent dye Fura-2 AM for 30 minutes prior to fluid flow.

Fluid Flow System Following incubation with Fura-2, the slides were mounted in a parallel plate flow chamber. A materials testing machine was used to pump a syringe, in series with rigid wall tubing and a flow meter, to drive oscillating fluid flow through the chamber. The flow media consisted of DMEM and 2% FBS. Cells were exposed to 2 minutes of oscillating fluid flow that produced a peak shear stress of 20 dynes/cm^2 at a frequency of 2 Hz. After a rest period of 5, 30, 60, 300, 600, 900, 1800, or 2700 seconds, the cells were exposed to a second bout of fluid flow. In a second set of experiments, different groups of cells were exposed to one hour of continuous oscillating flow.

Calcium Imaging The flow chamber was mounted on the stage of a fluorescent microscope. Images of the fluorescence intensity were collected during a no flow baseline period and the bouts of oscillating flow. Image analysis software (Metaflour, West Chester, PA) was used to determine real-time intracellular calcium concentrations ($[Ca^{2+}]_i$) using ratiometric dye methodology. $[Ca^{2+}]_i$ was determined for 25 individual cells for each slide. ANOVA was used to assess differences in the percentage of cells responding with $[Ca^{2+}]_i$ oscillations and the magnitude of the $[Ca^{2+}]_i$ oscillations between the two flow periods for each rest interval.

Results

With the onset of oscillating fluid flow there were immediate and transient $[Ca^{2+}]_i$ oscillations of 100 to 300 nM, which lasted approximately 60 seconds before returning to baseline values. While a small percentage of cells could respond to the second bout of fluid flow after only a 5 second rest period, significantly ($p < 0.04$) fewer cells displayed $[Ca^{2+}]_i$ oscillations when the rest period was less than 600 seconds (Figure 1). Additionally, the magnitude of the second $[Ca^{2+}]_i$ oscillation was significantly ($p < 0.01$) lower than the magnitude of the first $[Ca^{2+}]_i$ oscillation for rest periods less than 900 seconds (Figure 2). As many as four fluid flow induced $[Ca^{2+}]_i$ oscillations could be invoked when rest periods of 2700 seconds were given between each bout. However, during one hour of continuous oscillating fluid flow, no subsequent $[Ca^{2+}]_i$ oscillations were observed after the initial immediate response.

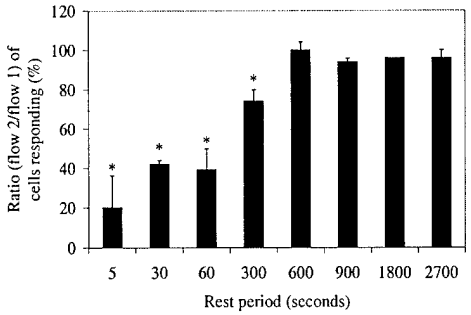

Figure 1: The percentage of cells displaying $[Ca^{2+}]_i$ oscillations during the second bout of flow was significantly lower (asterisks) than to the percentage of cells displaying $[Ca^{2+}]_i$ oscillations during the first bout of flow when the rest period was less than 600 seconds.

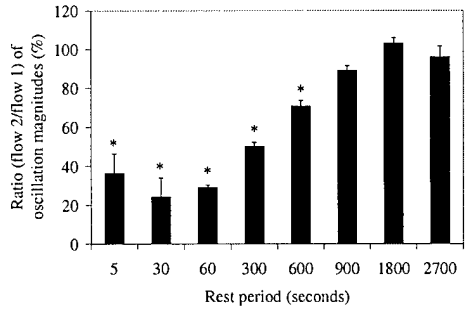

Figure 2: The magnitude of $[Ca^{2+}]_i$ oscillations during the second bout of flow was significantly lower (asterisks) than the magnitude of $[Ca^{2+}]_i$ oscillations during the first bout of flow when the rest period was less than 900 seconds.

Discussion

These findings suggest that a rest period is required for multiple fluid flow induced $[Ca^{2+}]_i$ responses in osteoblastic cells, but the refractory period may be as short as 5 seconds for some individual cells. However, a 900 second rest period was required to recover both the percentage of cells responding and the magnitude of the response. *In vivo*, rest periods enhance mechanically induced bone formation.[1] It is reasonable to hypothesize that $[Ca^{2+}]_i$ oscillations play a role in *in vivo* bone adaptation, possibly by influencing prostaglandin release or gene expression. Our findings may have important implications for the design and interpretation of future mechanotransduction experiments aimed at studying bone adaptation mechanisms.

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Temporal aspects of fluid flow induced intracellular calcium oscillations in osteoblastic cells

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Abstract

Mechanically driven fluid flow in bone is thought to be an important stimulus for bone cell mechanotransduction and bone adaptation. In vitro, short bouts of fluid flow cause rapid and transient increases in intracellular calcium concentration ($[Ca^{2+}]_i$) in osteoblastic cells. We studied the refractory period for $[Ca^{2+}]_i$ oscillations in primary rat osteoblastic cells during short-term fluid flow and the nature of multiple $[Ca^{2+}]_i$ oscillations during long-term flow. The cells were exposed to 2 minutes of oscillating fluid flow that produced shear stresses of 2 pascals at 2 Hz. After a rest period of 5, 30, 60, 300, 600, 900, 1800, or 2700 seconds, the cells were exposed to a second bout of flow. A 600 second rest period was required to recover the percentage of cells responding to fluid flow and a 900 second rest period was required to recover the $[Ca^{2+}]_i$ oscillation magnitude. The magnitude and shape of multiple $[Ca^{2+}]_i$ oscillations were strikingly similar for individual cells after a 900 second rest period. During 15 minutes of continuous oscillating flow, individual cells displayed between 1 and 9 oscillations subsequent to the initial response. However, only 54 % of the cells that responded initially displayed subsequent $[Ca^{2+}]_i$ oscillations and the magnitude of subsequent oscillations was only 28 % of the initial response. These findings may have important implications for downstream signaling events in osteoblastic cells during long-term fluid flow and for in vivo bone adaptation to mechanical loading.

Key Words

Mechanotransduction, osteoblast, calcium signaling, bone adaptation, oscillating fluid flow

Introduction

Bone adaptation to mechanical loading has been well documented in humans and other animals (N. Ashizawa, et al., 1999; H. Haapasalo, et al., 1996; L. E. Lanyon and C. T. Rubin, 1984; A. G. Robling, et al., 2000; C. T. Rubin, et al., 1995; C. H. Turner, et al., 1994). Currently, the biological mechanisms of bone adaptation are the focus of intense scientific inquiry. Knowledge of the cellular mechanisms involved in mechanically driven bone adaptation will likely underscore pharmaceutical therapies for many bone disorders (e. g., osteoporosis and fracture healing). Mechanically induced fluid flow within the lacuno-canalicular network of bone is believed to be an important bone cell stimulus for mediating bone adaptation to mechanical loading (E. H. Burger and J. Klein-Nulend, 1999; S. C. Cowin, et al., 1995; S. Weinbaum, et al., 1994). Fluid flow provides a mechanism for the transport of nutrients and waste products; it can also provide individual bone cells with information about the mechanical forces acting on whole bones. In vitro, bone cells respond to physical stimuli with a cascade of biological signaling events by a process known as mechanotransduction. One of the earliest events in bone cell mechanotransduction is intracellular calcium signaling. Fluid flow, which engenders cell membrane shear stress, induces a rapid and transient increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in osteoblastic cells (F. D. Allen, et al., 2000; N. X. Chen, et al., 2000; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998). Mechanically induced $[Ca^{2+}]_i$ oscillations have been shown to influence downstream events such as gene expression. Inhibiting $[Ca^{2+}]_i$ oscillations in osteoblastic cells inhibited fluid flow induced mRNA upregulation of the bone matrix protein osteopontin (J. You, et al., 2001).

In addition to influencing gene expression, Ca^{2+} signaling is implicated in numerous other cellular activities (e. g., proliferation, differentiation, and apoptosis) in many cell types (M.

J. Berridge, et al., 1998). However, the mechanisms for the pleiotropic actions of Ca^{2+} are only partially understood. The magnitude, duration, and frequency of $[Ca^{2+}]_i$ oscillations are all believed to play a role in regulating downstream events (A. P. Thomas, et al., 1996; E. C. Toescu, 1995). For example, varying the frequency of the $[Ca^{2+}]_i$ oscillations may be used to activate different genes (M. J. Berridge, et al., 1998), and the enzyme CaM Kinase II has been shown to effectively "count" $[Ca^{2+}]_i$ oscillations and vary its activity accordingly (P. De Koninck and H. Schulman, 1998). Agonists, which stimulate the release of calcium from the intracellular stores of smooth muscle cells, can induce $[Ca^{2+}]_i$ oscillations of 400 to 800 nM; the $[Ca^{2+}]_i$ oscillation frequency is dose-dependent, ranging between 4-30 oscillations per minute (J. P. Savineau and R. Marthan, 2000). Osteoblastic cells typically display a single $[Ca^{2+}]_i$ oscillation when exposed to short bouts (1.5 - 3 minutes) of fluid flow (C. T. Hung, et al., 1996; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998). However, multiple oscillations in some cells have been noted, but not characterized (C. T. Hung, et al., 1995).

The ability of ensembles of osteoblastic cells to respond to multiple bouts of fluid flow, separated by a 10 – 15 minute rest period, has been noted previously (C. T. Hung, et al., 1995). It was found that 58% of the cells responded to the initial loading bout and 45% responded to the second. The effect of rest periods, of varying durations, on Ca^{2+} signaling in osteoblastic cells has not been studied. Additionally, Ca^{2+} signaling in osteoblastic cells exposed to continuous loading of durations longer than 3 minutes has also not been studied previously. Yet, many in vitro mechanotransduction experiments use long-term (on the order of hours) bouts of fluid flow to study other biochemical responses such as prostaglandin release or gene expression (N. E. Ajubi, et al., 1999; J. You, et al., 2001). Since Ca^{2+} signaling is linked to the upregulation of

gene expression and prostaglandin release during long-term fluid flow, it will be important to elucidate the temporal aspects of Ca^{2+} signaling during long-term flow.

An understanding of the temporal aspects of Ca^{2+} signaling is also important for establishing relationships between in vitro mechanotransduction studies and in vivo adaptation phenomena. Histological changes in the architecture of bone have been characterized in animals exposed to unaccustomed mechanical loading. Partitioning a daily mechanical stimulus into discrete loading bouts enhances bone formation in rat tibiae (A. G. Robling, et al., 2000). This finding suggests that the bone cells involved in mediating mechanically induced bone adaptation have a refractory period, during which they are insensitive to additional mechanical stimuli. Bone formation was enhanced when 360 loading cycles were divided up into distinct bouts spaced over the course of a day, compared to a single bout of 360 cycles (A. G. Robling, et al., 2000). Based on these findings, we hypothesized that 1) a refractory period exists, during which time fluid flow induced $[Ca^{2+}]_i$ oscillations in bone cells are insensitive to additional bouts of fluid flow; and 2) that longer-term (15 minutes) continuous fluid flow produces multiple $[Ca^{2+}]_i$ oscillations in osteoblastic cells.

Method

Bone Cells

Rat osteoblastic cells (ROB) were isolated from the humeri, tibiae, and femora of 4-month-old male Fisher 344 rats. All procedures were approved by the Institutional Animal Care and Use Committee at the M. S. Hershey Medical Center. The rats were anesthetized by an intraperitonial injection of pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, IL) with a dosage of 50 mg/Kg of bodyweight and euthanized by exsanguination. The bones were extracted from the animals and subperiosteal ROB were obtained by removing all soft

tissues, including cartilage and periosteum, from the bones and performing sequential collagenase (Worthington Biochemical Corporation, Lakewood, NJ) digestions at 37°C. Cells from the first digestion were collected by centrifugation and discarded to eliminate any residual non-bone cells that were not removed by dissection. Cells from the second digestion were collected by centrifugation and grown to confluency in Dulbecco' Modified Eagle Medium (DMEM, Gibco, Gaithersburg, MD), 20% fetal bovine serum (FBS), and 1% penicillin/streptomycin. We have previously shown that ROB isolated by this technique display characteristics of the osteoblast phenotype (H. J. Donahue, et al., 1995). Three days prior to experimentation the cells were plated on quartz microscope slides (76 mm × 26 mm × 1.6 mm) at a density of 75,000 cells per slide; cells were approximately 80% confluent on the day of experimentation. The cells were incubated at 37°C with 10 μM Fura-2 AM (Molecular Probes, Eugene, OR) for 30 minutes prior to mechanical stimulation.

Fluid Flow System

Following Fura-2 loading the cell-seeded microscope slides were mounted in a parallel plate flow chamber, which was fixed to the stage of a fluorescent microscope. A fresh bolus of flow media was added to the chamber and the cells were left undisturbed for 30 minutes. The flow media consisted of DMEM and 2% FBS. We used a previously described fluid flow system to expose ROB to oscillating fluid flow (C. R. Jacobs, et al., 1998). To generate fluid flow induced shear stresses on the cells in the chamber, a materials testing machine was used to pump a syringe, which was in series with rigid wall tubing and a flow meter (Transonic Systems Inc., Ithaca, NY), driving fluid through the chamber. This system produces laminar fluid flow in the chamber with an oscillating flow profile. Shear stresses on the chamber walls are dependent on the chamber dimensions and the rate of fluid flow (C. T. Hung, et al., 1995). Thus, we were able

to generate shear stresses on the cells with magnitudes that they are predicted to experience *in vivo* (S. Weinbaum, et al., 1994). Oscillating fluid flow was used because it more closely simulates physiologic bone loading than steady or pulsatile flow (C. R. Jacobs, et al., 1998). During experimentation the cells were exposed to 2 minutes of oscillating fluid flow that produced shear stresses of 2 pascals (Pa) at a frequency of 2 Hz. After a rest period of 5, 30, 60, 300, 600, 900, 1800, or 2700 seconds, the cells were exposed to a second bout of fluid flow. In a second set of experiments, different groups of cells were exposed to 15 minutes of continuous oscillating flow.

Calcium Imaging

Real-time $[Ca^{2+}]_i$ was quantified using ratiometric dye methodology. When Fura-2 binds Ca^{2+}, its maximal absorption wavelength shifts from 363 nm for Ca^{2+}-free Fura-2 to 335 nm for Ca^{2+}-bound Fura-2 (A. Takahashi, et al., 1999). In practice, wavelengths of 340 and 380 nm are used for ratiometric measurements. The emission peak is near 510 nm for both Ca^{2+}-free and Ca^{2+}-bound Fura-2. ROB cell ensembles were illuminated at wavelengths of 340 and 380 nm; emitted light was passed through a 510 nm filter and images were collected with a CCD camera. Images of fluorescence intensities were collected every two seconds for a one minute no flow period (baseline) and during the 2 minute bouts of oscillating fluid flow. $[Ca^{2+}]_i$ was determined from the ratio of the two emission intensities using calibrated standards and image analysis software (Metaflour, West Chester, PA). Temporal $[Ca^{2+}]_i$ profiles were determined for at least 50 individual cells for each rest period. We defined a responsive cell as one that displayed a transient increase in $[Ca^{2+}]_i$ of at least 4-fold the maximum oscillation value recorded during the baseline period. We assessed the percentage of cells responding with a $[Ca^{2+}]_i$ oscillation and the magnitude of the $[Ca^{2+}]_i$ oscillations.

Statistics

One-way ANOVA's were used to look for differences, between the first and second loading bouts, in the percentage of cell responding and the magnitude of the response for each rest period using Statview software (SAS Institute, San Francisco, CA). Because of heterogeneity in $[Ca^{2+}]_i$ responses, the magnitude of the $[Ca^{2+}]_i$ oscillations and the percentage of cells responding are presented graphically as the ratio of the oscillation that occurred during the second bout of fluid flow to the oscillation that occurred during the first bout of fluid flow to facilitate comparisons between rest period groups. For the continuous loading regime, ANOVA's were used to compare the percentage of cell responding and the magnitude of the response between the first and subsequent oscillations. A significance level of 0.05 was used for all statistical analyses.

Results

With the onset of the first bout of fluid flow there were rapid and transient increases in $[Ca^{2+}]_i$, which lasted approximately 60 seconds (Fig. 1). Peak values were reached approximately 15 seconds after the onset of fluid flow. During the first bout of fluid flow, the mean ($\pm$ SE) percentage of cells displaying $[Ca^{2+}]_i$ oscillations was 96.7 $\pm$ 1.3 % with a mean ($\pm$ SE) magnitude of 185 $\pm$ 6 nanomolar (nM). Some cells could respond to a second bout of fluid flow after only a 5 second rest period; however, only 20 % of the cells that responded during the first bout could respond during the second bout (Fig. 2). The percentage of cells responding to the second bout of flow was significantly ($p < 0.049$) lower than the percentage responding to the first bout for rest periods less than 10 minutes (Fig. 2). For rest periods of 10 minutes or longer there were no significant ($p > 0.095$) differences in the percentage of cells responding between the two bouts of fluid flow.

Although some cells could respond to the second bout of flow after only a 5 second rest period, the magnitude of the $[Ca^{2+}]_i$ oscillation during the second bout of fluid flow was only 36 % of the oscillation magnitude during the first bout of flow (Fig. 3). The magnitude of the second $[Ca^{2+}]_i$ oscillation was significantly ($p < 0.008$) lower than the magnitude of the first $[Ca^{2+}]_i$ oscillation for rest periods less than 15 minutes (Fig. 3). After a 15 minute rest period, 94 $\pm$ 2 % of the cells that responded to the first bout of flow responded to the second bout with 89 $\pm$ 3 % of the magnitude of the first $[Ca^{2+}]_i$ oscillation. Thus, a 15 minute rest period between loading bouts was required to regain both the percentage of cells responding and the magnitude of the $[Ca^{2+}]_i$ oscillations.

Not only was there no significant difference in the oscillation magnitude after at least a 15 minute rest period, there was a striking similarity in the multiple $[Ca^{2+}]_i$ oscillation profiles of individual cells. If individual cells responded to the first bout of fluid flow, they typically responded to the second bout with the same magnitude $[Ca^{2+}]_i$ oscillation (Fig. 4). If cells did not respond to the first loading bout, they typically did not respond to the second (Fig. 4). Furthermore, not only was there a striking similarity in the $[Ca^{2+}]_i$ oscillation magnitudes for multiple oscillations in individual cells, there were remarkable similarities in the shape and duration of the $[Ca^{2+}]_i$ oscillation profiles (Fig. 5).

During 15 minutes of continuous oscillating fluid flow many cells displayed multiple $[Ca^{2+}]_i$ oscillations. Individual cells displayed between 1 and 9 $[Ca^{2+}]_i$ oscillations subsequent to the initial response (Figs. 6a and b), with a mean of 3.9 $\pm$ 0.5 subsequent oscillations. 100 % of the cells exposed to 15 minutes of continuous oscillating fluid flow displayed an initial response. However, only 54 % of the cells that responded initially displayed subsequent $[Ca^{2+}]_i$ oscillations

(Fig. 7). Furthermore, the magnitude of the subsequent $[Ca^{2+}]_i$ oscillations were only 28 % of the initial response (Fig. 8).

Discussion

While it is clear that bones adapt to their mechanical loading environment, it is unclear how bone cells accomplish adaptation phenomena. In vitro, bone cells respond to physical stimuli with numerous biochemical responses. However, the details of the mechanotransduction signaling pathways are only beginning to emerge. It is well established that $Ca^{2+}{}_i$ signaling in osteoblastic cells is one of the earliest responses to fluid flow (F. D. Allen, et al., 2000; N. X. Chen, et al., 2000; C. T. Hung, et al., 1996; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998). It has also been demonstrated that $Ca^{2+}{}_i$ signaling is required for downstream events such as the upregulation of osteopontin mRNA during long-term fluid flow.(J. You, et al., 2001) However, the nature of the temporal aspects of $Ca^{2+}{}_i$ signaling during long-term flow and how they mediate other components of the signaling cascade are unclear. Since bone cells engender whole bone adaptations to mechanical loading it is likely that some, if not all, of the components of the mechanotransduction signaling cascade have similar temporal characteristics as mechanically induced bone formation in whole bones. When a mechanical stimulus was partitioned into distinct loading bouts, periosteal bone formation was enhanced in rat tibiae, suggesting the existence of a refractory period in the mechanotransduction signaling cascade of bone cells (A. G. Robling, et al., 2000). Therefore, we studied the refractory period for $[Ca^{2+}]_i$ oscillations in bone cells exposed to distinct bouts of fluid flow and the nature of multiple $[Ca^{2+}]_i$ oscillations during continuous long-term flow.

$[Ca^{2+}]_i$ oscillations are associated with the activation of numerous $Ca^{2+}{}_i$-dependent enzymes which are involved in many different cellular functions (M. J. Berridge, et al., 1998).

With the onset of a $[Ca^{2+}]_i$ oscillation there is also an immediate activation of processes that restore $[Ca^{2+}]_i$ to resting levels to prevent extended exposure to toxic levels of $[Ca^{2+}]_i$ (E. C. Toescu, 1995). During the $[Ca^{2+}]_i$ oscillation, cytosolic Ca^{2+} is rapidly buffered by many different molecules such as nucleotides, organic acids, and proteins, which alters the diffusion coefficient of cytosolic Ca^{2+} (E. C. Toescu, 1995). Cytosolic Ca^{2+} is also pumped out of the cell and back into intracellular stores by Ca^{2+}-ATPases. The rates of buffered Ca^{2+} diffusion in the cytosol and the kinetics of refilling intracellular Ca^{2+} stores may determine the refractory period for fluid flow induced $[Ca^{2+}]_i$ oscillations. We found that a 10 minute rest period was required to recover the percentage of cells responding to a bout of fluid flow and a 15 minute rest period was required to recover the magnitude of the $[Ca^{2+}]_i$ oscillations. These periods may represent the time intracellular Ca^{2+} stores need to regain their homeostatic $[Ca^{2+}]$ so that they are ready for another signaling event.

Hung et al. (1995) found that 58% of cells responded to an initial bout of fluid flow and 45% responded to a second bout after a 10-15 minute rest period, but most of the cells responding to the second bout had not responded to the first bout. This is inconsistent with our finding that cells which displayed oscillations typically did so in both flow periods for rest periods of 10 minutes or longer. There are many possible explanations for this discrepancy. For example, they used primary cells isolated from neonatal rat calvaria and a serum-free perfusate with a steady flow profile. Indeed, it has been demonstrated that cells display differential calcium responses to oscillating and steady flow (C. R. Jacobs, et al., 1998). The results of Hung et al. (1995) may be viewed as the refractory nature of a pure mechanotransduction response to steady flow because of the absence of serum. Whereas our findings may reflect a chemotransduction response or an integrated chemo/mechanotransduction response to oscillating

flow. An important note on our data is worth consideration as it represents a limitation in determining cellular responses for the shorter rest periods (i. e., $\leq$ 1 minute). Immediately following the return of the initial $[Ca^{2+}]_i$ oscillations to baseline values, the $[Ca^{2+}]_i$ profiles displayed more noise or low magnitude fluctuations than they did during the initial baseline period (fig 1). Therefore the cells that were quantified as responders to the second bout of flow, for rest periods of 1 minute or less, may have actually still been fluctuating due to the initial bout rather than responding, per se, to the second bout. In that case the values of the percentage of cells responding and the magnitude of the response that were presented for these short rest periods may actually be even smaller.

The heterogeneity of $[Ca^{2+}]_i$ responses to fluid flow and agonist stimulation has been previously noted.(R. Civitelli, 1992; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998) Individual cells display unique combinations of $[Ca^{2+}]_i$ oscillation characteristics such as the magnitude, rise and fall times, duration, and overall shape. These characteristics define what has been termed a "Ca^{2+} fingerprint" (M. Prentki, et al., 1988). The unique $[Ca^{2+}]_i$ oscillation profiles of individual cells, stimulated by the agonist carbamylcholine, were almost identically reproduced when the cells were restimulated carbamylcholine (M. Prentki, et al., 1988). Hung et al. (1995) proposed that shear stress gradients may contribute to the heterogeneity of fluid flow induced $[Ca^{2+}]_i$ oscillations. However, the addition of the calcium agonist bradykinin, in the absence of flow, also produced a heterogeneous response (C. T. Hung, et al., 1995). Prentki et al. (1988) suggested that the Ca^{2+} fingerprint is an intrinsic property of individual cells, which is independent of stochastic events and allows them to respond in a selective and reproducible manner. Indeed, the reproducible profiles of multiple $[Ca^{2+}]_i$ oscillations in individual

osteoblastic cells, exposed to multiple bouts of fluid flow (fig 5), suggest that Ca^{2+} fingerprints exist in osteoblastic cells and may have a role in mechanotransduction.

Bone cells typically display a single $[Ca^{2+}]_i$ oscillation when exposed to short bouts (1.5 - 3 minutes) of steady, pulsatile, or oscillating fluid flow.(C. T. Hung, et al., 1996; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998) However, multiple oscillations have been noted during short bouts of flow (C. T. Hung, et al., 1995). We found that during 15 minutes of continuous oscillating fluid flow, distinct $[Ca^{2+}]_i$ oscillations could occur as shortly as 60 seconds after the initial $[Ca^{2+}]_i$ oscillations had returned to baseline values. As many as 9 subsequent $[Ca^{2+}]_i$ oscillations were observed in individual bone cells during the 15 minute loading period. However, the percentage of cells displaying subsequent oscillations and the magnitude of the subsequent $[Ca^{2+}]_i$ oscillations were significantly lower than the initial $[Ca^{2+}]_i$ responses. It is possible that the subsequent low magnitude and low frequency $[Ca^{2+}]_i$ oscillations encode a different biochemical message than the initial response. Indeed, low frequency $[Ca^{2+}]_i$ oscillations may be integrated into a potent biological message over time, such as incremental Ca^{2+}-dependent phosphorylation of regulatory proteins (J. W. Putney, Jr. and G. S. Bird, 1993). The finding that some cells display multiple $[Ca^{2+}]_i$ oscillations during long-term oscillating flow may have important implications for downstream events such as the upregulation of gene expression. For example, two hours of continuous oscillating fluid flow increased osteopontin gene expression 4-fold over no flow control levels; however, this increase was prevented when cells were treated with the Ca^{2+}-ATPase inhibitor thapsigargin to prevent the release of Ca^{2+} from intracellular stores (J. You, et al., 2001). It is reasonable to hypothesize that multiple $[Ca^{2+}]_i$ oscillations during long-term flow influence gene expression by acting on Ca^{2+}-dependent enzymes such as CaM Kinase II.

Clearly bone cells display two distinct calcium responses to fluid flow. The first is the immediate response that occurs with the onset of flow, in which individual cells display large heterogeneity in the $[Ca^{2+}]_i$ oscillation magnitude, but relative homogeneity in the time to activation and duration of the $[Ca^{2+}]_i$ oscillation. This response can be reproduced, with striking similarity in the $[Ca^{2+}]_i$ profiles of individual cells, when rest periods greater than 15 minutes are inserted between loading bouts. The second type of response is the multiple $[Ca^{2+}]_i$ oscillations that occur subsequent to the initial response during long-term fluid flow. These subsequent $[Ca^{2+}]_i$ oscillations have lower magnitudes than the initial responses and display large heterogeneity in the number of subsequent oscillations that occur in individual cells as well as in the oscillation magnitude. Our findings raise the possibility that multiple, low amplitude $[Ca^{2+}]_i$ oscillations are involved in regulating the downstream responses of bone cells to long-term fluid flow such as gene expression. Partitioning a daily mechanical stimulus into discrete loading bouts enhances bone formation in rat tibiae (A. G. Robling, et al., 2000). Our finding that the magnitude of the $[Ca^{2+}]_i$ oscillation and the percentage of cells responding could be regained, when rest periods of at least 15 minutes were inserted between loading bouts, supports a role for Ca^{2+} signaling in bone adaptation to mechanical loading. For cell processes where the oscillation magnitude and percentage of cells responding are important, rest periods may increase the number of times those processes are activated.

Acknowledgements

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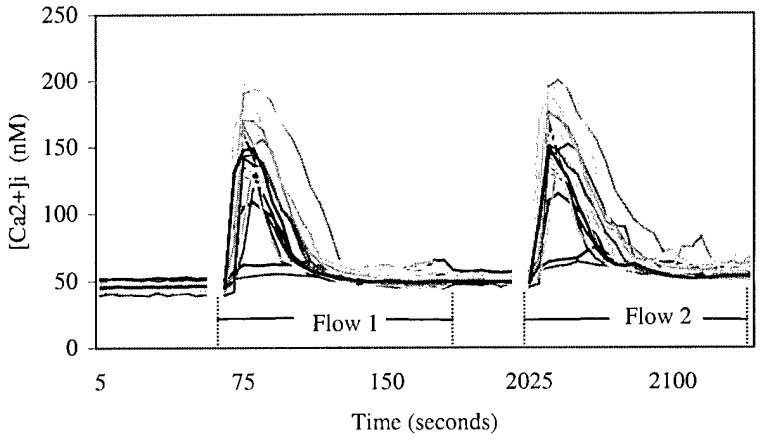
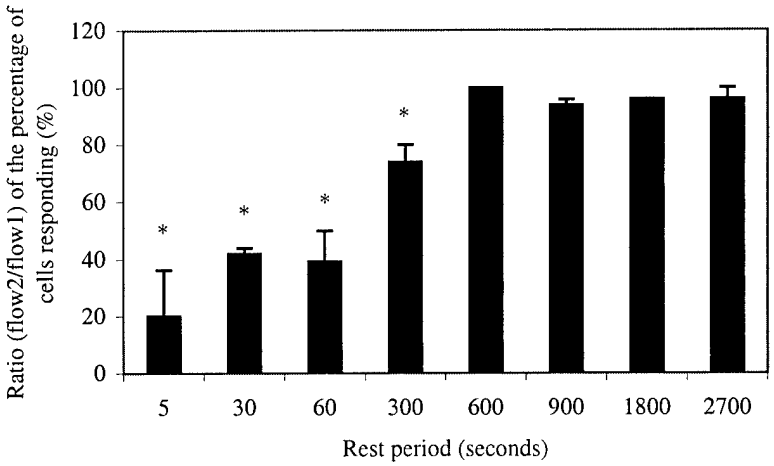
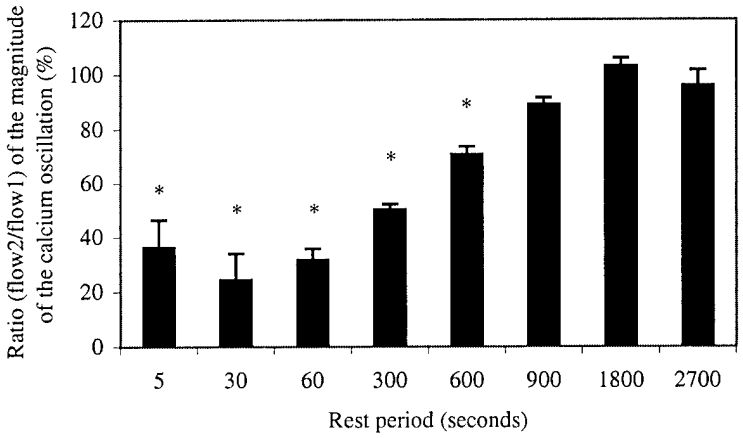
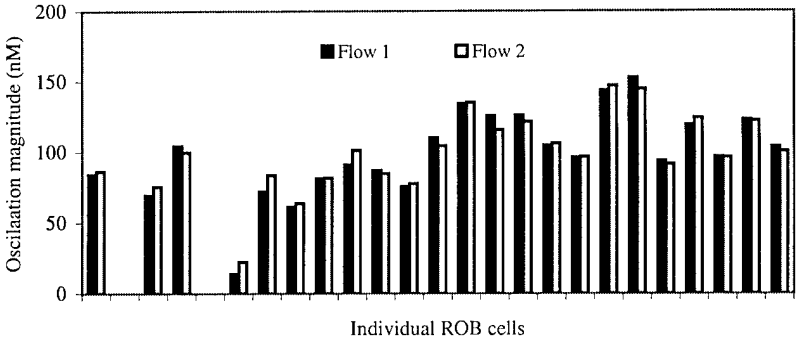
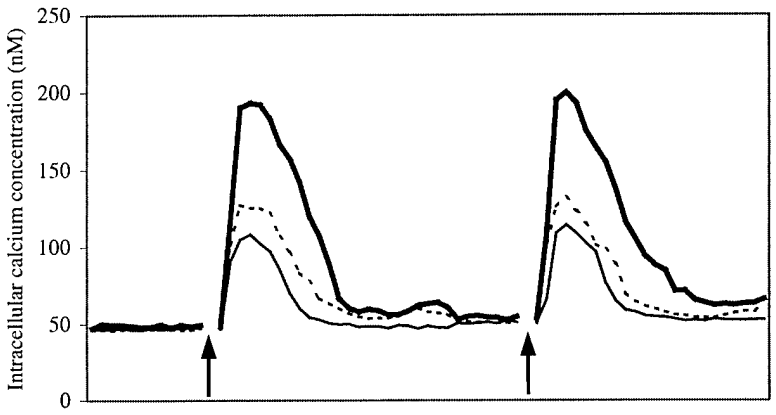

250
200
150
100
50
0
[Ca2+]i (nM)
Flow 1
Flow 2
5
75
150
2025
2100
Time (seconds)

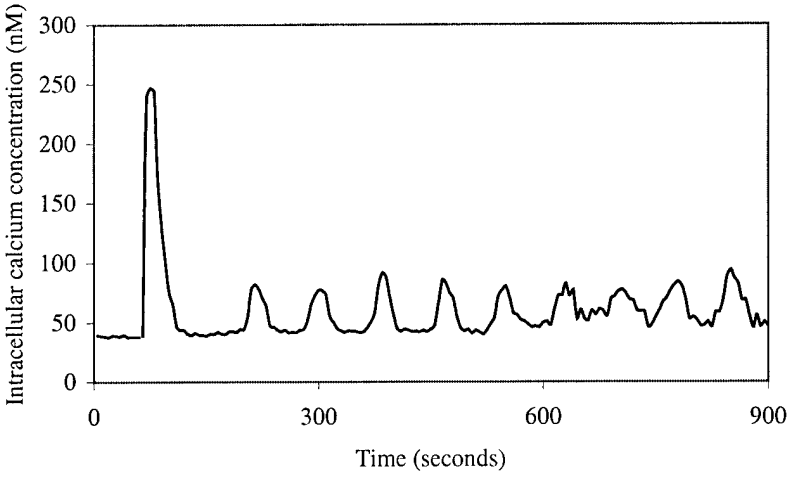

Ratio (flow2/flow1) of the percentage of cells responding (%)
120
100
80
60
40
20
0
*
*
*
*
5
30
60
300
600
900
1800
2700
Rest period (seconds)

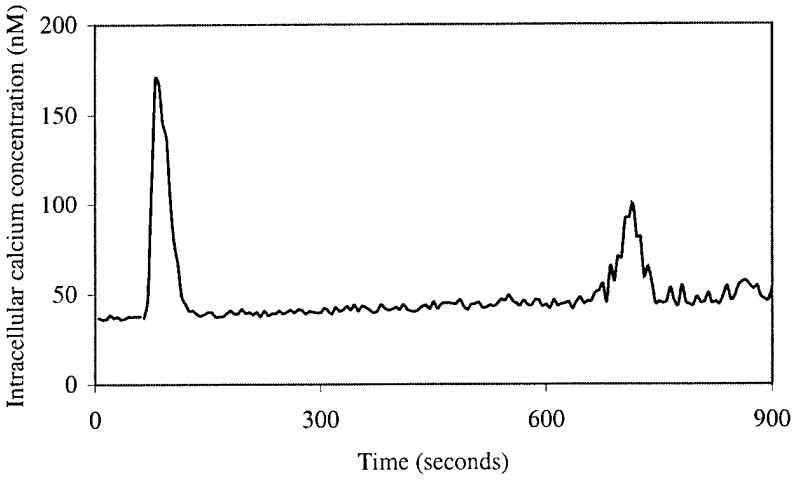

Ratio (flow2/flow1) of the magnitude of the calcium oscillation (%)
120
100
80
60
40
20
0
*
*
*
*
*
5
30
60
300
600
900
1800
2700
Rest period (seconds)

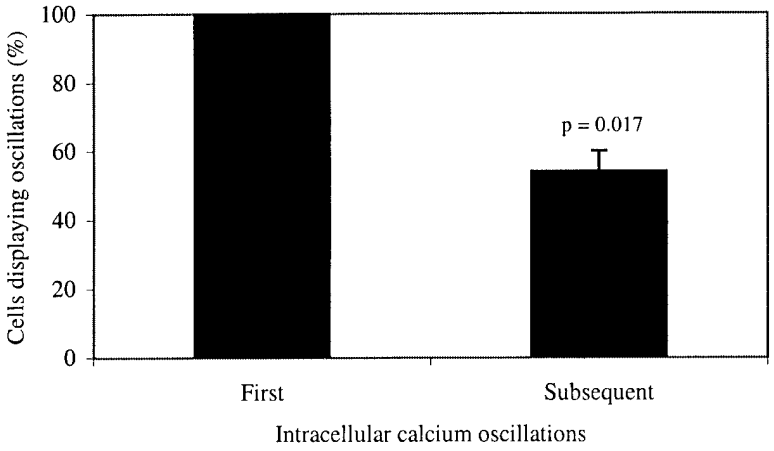

200
150
100
50
0
Oscilaation magnitude (nM)
Flow 1
Flow 2
Individual ROB cells



2-minute bouts of fluid flow separated by a 30 minute rest period


Intracellular calcium concentration (nM)
300
250
200
150
100
50
0
0
300
600
900
Time (seconds)


Intracellular calcium concentration (nM)
200
150
100
50
0
0
300
600
900
Time (seconds)


100
80
60
40
20
0
Cells displaying oscillations (%)
p = 0.017
First
Subsequent
Intracellular calcium oscillations

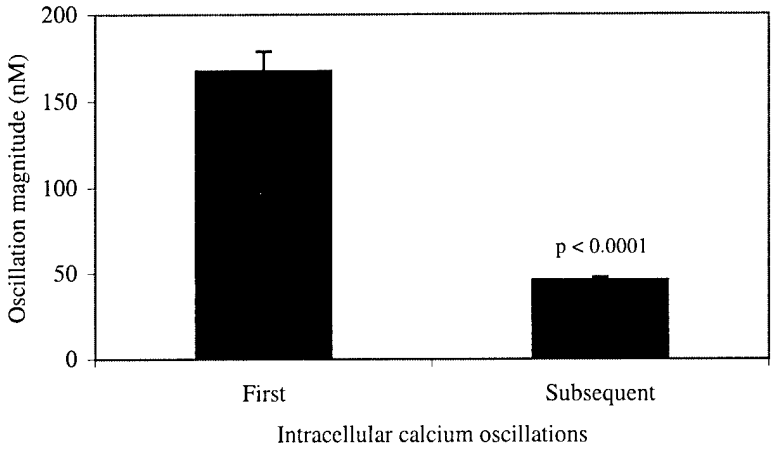

200
150
100
50
0
Oscillation magnitude (nM)
p < 0.0001
First
Subsequent
Intracellular calcium oscillations

Figure Legend

Fig. 1. Representative $[Ca^{2+}]_i$ profiles of 25 individual cells for two 2-minute bouts of oscillating fluid flow separated by a 30 minute rest period.

Fig. 2. The percentage of cells responding to the second bout of flow normalized by the percentage of cells responding to the first bout of flow. For rest periods less than 10 minutes significantly ($p < 0.05$; denoted by the asterisks) fewer cells responded to the second bout of flow than to the first bout.

Fig. 3. The magnitude of the second $[Ca^{2+}]_i$ oscillation normalized by magnitude of the first $[Ca^{2+}]_i$ oscillation. For rest periods less than 15 minutes the oscillation magnitude was significantly ($p < 0.05$; denoted by the asterisks) lower during the second bout of flow during the first bout.

Fig. 4. The $[Ca^{2+}]_i$ oscillation magnitudes of 23 individual cells exposed to two bouts of fluid flow separated by a 30 minute rest period. Individual cells displayed a remarkable similarity in the oscillation magnitudes of two distinct loading bouts for a wide range of concentration values. The two cells that did not respond, did not respond during either loading bout.

Fig. 5. Demonstration of the "Ca^{2+} fingerprint" in three individual osteoblastic cells. Individual cell $[Ca^{2+}]_i$ profiles showed striking similarity in the magnitude, duration, and overall shape when stimulated by multiple fluid flow events. Arrows indicate the onset of flow.

Fig. 6. Individual osteoblastic cells displayed between 1 (a) and 9 (b) lower magnitude oscillations after the initial response when exposed to 15 minutes of continuous oscillating fluid flow.

Fig. 7. A significantly smaller percentage of cells displayed subsequent oscillations during the 15 minutes loading bout than the percentage of cells that displayed an initial response at the onset of flow.

Fig. 8. During the 15 minutes loading bout, the magnitude of the $[Ca^{2+}]_i$ oscillations was significantly lower in the subsequent oscillations than it was in the initial oscillation.